



Enzymatic Production of Ceramide

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Enzymatic Production of Ceramide

Long Zhang

Ph.D. thesis

October 2008

Center for Biological Sequence Analysis

Department of Systems Biology

Technical University of Denmark

Preface

The present thesis entitled: “Enzymatic production of ceramide” is part of the requirements for the acquirement of a PhD degree in biotechnology at the Technical University of Denmark (DTU). This study was performed primarily at the Department of Systems Biology-DTU and the National Food Institute-DTU. The work was carried out under the supervision of Associate Professor Dr. Lars I. Hellgren and Professor Dr. Xuebing Xu, who was the main supervisor before he moved to the University of Aarhus. One part of the study was also conducted under the supervision of Associate Professor Dr. Gunnar Eigil Jonsson from the Department of Chemical and Biochemical Engineering-DTU. The PhD project was started on the 1st of March, 2005. I have suspended my PhD studies from the 1st of August, 2006, to the 31st of December, 2006, in order to join the Human Milk Fat Substitute Project, which was under the supervision of Professor Dr. Xuebing Xu.

The PhD project was financed primarily by the Technical University of Denmark for a period from 2005 to 2008. The study was also partially supported by the Center for Advanced Food Studies (LMC). A Lipidforum doctorate student grant was received to support the participation of the 5th Euro Fed Lipid Congress and 24th Nordic Lipid Symposium, Gothenburg, Sweden, in 2007.

October, 2008
Lyngby, Denmark

Long Zhang

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Melanie Giron, Shanshan Liang, Kasper Skov and Kristine Weirum are thanked for good work in their student projects. The list of people that should be thanked for their support during my three years of the study is long. In order to avoid a 5 page long list of the names, I will summarize in one sentence: I appreciate every teacher, employee and administrator at DTU for contributing to a happy experience and good study environment.

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Summary

Ceramide and ceramide derivatives have attracted large interest as active components in both pharmaceutical and cosmetic industries. This project is a study on the enzymatic production of ceramide. The main part of this thesis focused on the production of ceramide through enzymatic hydrolysis of sphingomyelin (SM). Natural SM from different sources was hydrolyzed through catalysis with liquid enzyme, enzyme immobilized on particle carriers, and enzyme immobilized on membranes. Two-phase (water: organic solvent) systems were commonly used for the reactions, with the reaction volume ranging from 0.2 ml to 79 ml. In the evaluation of the effects from multiple parameters, the principles of response surface methodology (RSM) were applied to assist the experimental design and data analysis. The primary analytical method employed was high performance thin-layer chromatography. Another part of this thesis was on the enzymatic synthesis of the structure-specific ceramides.

In a single-batch reaction, SM hydrolysis was more efficient in a two-phase (water: organic solvent) system rather than in a one-phase (water-saturated organic solvent) system. Among the enzymes screened, phospholipase C (PLC) from *C. perfringens* was found to have the most advantageous properties. Addition of ethanol to the system enhanced the rate of SM hydrolysis, and a mixture of ethyl acetate and hexane (50:50 in Vol.) was found to be the best organic system tested. When the hydrolysis was optimized using RSM with five factors, water amount and enzyme amount had the most significant effects on the hydrolysis reaction in the fitted quadratic model. SM hydrolysis using Tris-HCl buffer had a higher reaction rate than using a mixture buffer having a broad range from pH 2 to pH 12. Increasing the concentration of Tris-HCl buffer had a negative effect on SM hydrolysis.

To improve the reusability of the enzyme, the immobilization of PLC and the properties of the immobilized enzyme were studied. In the screening of enzyme carriers, Lewatit VP OC 1600 (Lew) showed the best performance in terms of activity. Pre-wetting Lew with ethanol led to more enzyme being fixed on the carrier, but the activity of the enzyme was decreased. Increasing the initial enzyme concentration resulted in more enzyme loading on the carrier and a higher specific activity. The

reaction catalyzed by the immobilized enzyme had an optimal temperature around 46 °C and optimal water content 3.5 %. The reaction had little dependence on pH. After 7 cycles, the immobilized enzyme retained around 70 % of its initial activity. When magnetic particles were used in the immobilization, the specific activity of the immobilized PLC was significantly decreased.

In the kinetic study of SM hydrolysis, thermal inactivation of the enzyme in solution, the enzyme powder and the enzyme immobilized on particle carriers did not follow the first order reaction at heating temperature 65 °C. The reactions for both the soluble and immobilized PLC followed Michaelis-Menten kinetics. The value of V_{\max} was markedly decreased by the immobilization without much change in K_m , as if the immobilization resulted in non-competitive inhibition. Ceramide product activated the hydrolysis reaction; however, the kinetic study proved that its addition mainly caused an increase in the affinity of the enzyme-substrate complex.

In the membrane reactor development for SM hydrolysis, the aqueous and organic phases were separated by a membrane containing the immobilized enzyme, while the organic phase was continuously circulated. Among ten membranes tested, the enzyme immobilized in membrane RC 70PP retained the highest activity with low immobilization efficiency. When three immobilization methods, filtration, covalent binding, and cross-linking, were compared, the enzyme immobilized by filtration had the highest activity with low immobilization efficiency. The optimal flow rate of the organic phase was 5 ml/min. The immobilized enzyme retained 16 % of its original activity after 5 cycles. The enzyme membrane reactor offered the improved enzyme reusability, the fast immobilization process, the straightforward up-scaling and the ability to combine the hydrolysis with the product separation.

Several schemes had been proposed for the synthesis of more structurally-complicated ceramides. All schemes included a key step, the enzymatic acylation of sphingosine. This step was proven to be a viable method for the production of simpler-structure ceramide in lab scale. A 50 % yield was achieved after 24 h reaction time.

Overall in the present study, efficient and scalable methods have been developed for the enzymatic production of ceramide and essential, novel and detailed information for industrial applications has been provided.

Sammenfatning

Ceramid og ceramiderivat har skabt stort interesse som aktive komponenter både indenfor lægemiddels og kosmetikindustrien. Dette projekt er et studie af enzymatisk produktion af ceramid. Hoveddelen af afhandlingen fokuserer på enzymatisk hydrolyse af sphingomyelin (SM). Naturlig SM fra forskellige kilder er blevet hydrolyseret ved hjælp af enzym opløst i vandig opløsning, enzym immobiliseret til partikulære carriers og enzym immobiliseret til membran. Der er hovedsagligt brugt et to-fas system (vand:organiske solvent) til de enzymatiske reaktioner og de brugte reaktionsvolumen varierede mellem 0,2 til 79 ml. Effekten af flere forskellige parametre på reaktionseffektivitet blev undersøgt ved at applicere response surface methodology (RSM) i den eksperimentelle design og data analyse. High performance thin-layer chromatography er blevet brugt som hovedsagelig kemiske analysemetode. I afhandlingen indgår også et afsnit om syntese af særskilte ceramidstrukturer.

Når reaktionen skedte som en single-batch reaction, var SM hydrolyse effektivere i et to-fas system (vand:organisk) end i et en-fase system (vand-mættet organisk solvent).. Ud af de undersøgte enzymer, viste sig phospholipase C (PLC) fra *C. perfringens* have mest gunstige egenskaber. Tilsætning af ethanol til system øgede SM hydrolyse hastigheden og en blanding af ethylacetat og hexan (50:50 af vol.) viste sig være det bedste organiske system. Når hydrolysen blev optimeret med hensyn til 5 faktorer vha RSM havde mængden vand og enzym i systemet, den mest signifikante effekt på hydrolysehastighed i en ”fitted quadratic model. Reaktionshastigheden af SM hydrolyse var hurtigere i en Tris-HCl end i en buffer blanding som kunne benyttes i et meget bredt pH-interval (2-12). En forøget koncentration af Tris-HCl havde dog en negativ indvirkning på SM hydrolyse reaktionen.

For at forbedre mulighederne at genbruge enzymet, undersøgte immobilisering af PLC samt det immobiliserede enzyms egenskaber. I en undersøgelse af forskellige carriers, havde det immobiliserede enzyme højst aktivitet på Lewatit VP OC 1600 (Lew). Det var muligt at forøge mængden bundet enzym på Lew partiklerne, men samtidigt mindskede enzymaktiviteten. Mere enzym blev bundet til partiklerne, når den initiale enzymkoncentration øgedes, og samtidigt steg den specifikke aktivitet. Den optimale temperatur for katalyse af reaktionen var omkring 46 °C og det optimale

vandindhold var 3.5 %. Reaktionen havde en meget lav pH afhængighed. Efter 7 cykler, bibeholdt det immobiliserede enzym omkring 70 % af den initiale aktivitet. Ved immobilisering til magnetiske partikler, faldt reaktionshastigheden markant.

Varmeinaktivering af enzymet ved 65 °C fulgte ikke første ordningens kinetik i opløsning, som puder eller når det var immobiliseret til partikulære carriers. Både opløst og immobiliseret PLC fulgte Michaelis-Menten kinetik. Immobiliseringen sænkede V_{\max} markant, uden at føre til en nævneværdig forandring af K_m . som om immobiliseringen førte til en non-kompetitiv inhibering. Produktet, ceramid, aktiverede hydrolysereaktionen, studierne af kinetikken viste at dette primært skyldes en øget affinitet i substrat-enzym komplekset.

Ved udviklingen af en membranreaktor til SM hydrolyse, separeredes den vandige og organiske fase af en membran som indholdt de immobiliserede enzym, mens den organiske fase cirkulerede kontinuerligt. Blandt de ti membraner som blev afprøvede, bibeholdt enzymet i membranen RC 70PP, men med lav immobiliserings effektivitet.

Når tre immobiliserings metoder, filtrering, kovalent binding samt cross-linking sammenlignedes, havde enzym som var immobiliseret ved filtrering den højeste aktivitet med lav immobiliserings effektivitet. Den optimale cirkulationshastighed for den organiske fase var 5 ml/min. Efter fem reaktionscykler, bibeholdt enzymet 16 % af den oprindelige aktivitet. Membranreaktoren forbedrede muligheden at genbruge enzymen, havde en hurtig immobiliserings proces, er let at opskalere og gjorde det muligt at kombinere hydrolysereaktionen med produktseparation.

Flere forskellige mulige metoder til syntese af mere komplicerede ceramider foreslås. Samtlige metoder indeholder enzymatisk acylering af sphingosin som et nøgle-trin. Der blev vist at dette er en mulighed ved brug af et rekombinant humant neutralt ceramidase. Efter 24 timers reaktionstid opnåedes et udbytte på 50 %.

Sammenfatningsvis har dette studie ført til udvikling af en effektiv og skalerbar metode til enzymatisk production af ceramid og vigtig, ny og detaljeret viden som kan bruges til industrielle applikationer er blevet genereret.

List of publications

This thesis is based on the following scientific papers.

- I. Long Zhang, Lars I. Hellgren and Xuebing Xu (2006) Enzymatic production of ceramide from sphingomyelin, *Journal of Biotechnology*, 123: 93–105
- II. Long Zhang, Lars I. Hellgren and Xuebing Xu (2007) Immobilization of phospholipase C for the production of ceramide from sphingomyelin hydrolysis, *Journal of the American Oil Chemists Society*, 84: 237-247
- III. Long Zhang, Lars I. Hellgren and Xuebing Xu (2008) Kinetic study of sphingomyelin hydrolysis for ceramide production, *Journal of Molecular Catalysis B: Enzymatic*, 51: 93-99
- IV. Long Zhang, Shanshan Liang, Lars I. Hellgren, Gunnar Eigil Jonsson and Xuebing Xu (2008) Phospholipase C-catalyzed sphingomyelin hydrolysis in a membrane reactor for ceramide production, *Journal of Membrane Science*, 325: 895–902

Abbreviations

BLM:	Bacitracin-linked magnetic particles
BSE:	Bovine spongiform encephalitis
DAG:	diacylglycerol
HPTLC:	High performance thin-layer chromatography
Lew:	Lewatit VP OC 1600
M-PVA N12:	Poly(vinyl alcohol) amino-activated magnetic beads
MWCO:	Molecular weight cut-off
PC:	Phosphatidylcholine
PC-PLC:	Phosphatidylcholine-specific Phospholipase C from <i>Bacillus cereus</i>
PI-PLC:	Phosphatidylinositol-specific Phospholipase C from <i>Bacillus cereus</i>
PLA ₂ :	Phospholipase A ₂
PLC:	Phospholipase C
PLD:	Phospholipase D
RSM:	Response surface methodology
SM:	Sphingomyelin
SMase:	Sphingomyelinase
<i>sn</i> :	Stereospecific numbering
TAPS:	tetraacetylphytosphingosine
TLC:	Thin-layer chromatography
Tris:	Tris(hydroxymethyl)amino-methan
U:	Unit, one unit will liberate 1.0 μ mole of water soluble organic phosphorus from the preferred substrate of the enzyme per min under certain conditions

1 Introduction

Ceramide, the N-acylated derivative of sphingosine, is the key intermediate in the biosynthesis of all complex sphingolipids. Due to its major role in maintaining the water-retaining properties of the epidermis (Coderch *et al.*, 2003), ceramide has great commercial potential in cosmetic and pharmaceutical industries for uses in hair and skin care products. Many products containing ceramide or its synthetic analogues have already been introduced in the cosmetic market, and the effect of its application is excellent. Application of 0.1 % ceramide in a hydrophilic ointment was found to have good healing effects on patients of atopic dermatitis (Hara *et al.*, 1999). Promising results of a phase 1 trial of a ceramide-dominant, barrier repair moisturizer have been reported in childhood atopic dermatitis (Chamlin *et al.*, 2001). Ceramide-containing products are also effective in restoring the water content of dry skin and in relieving atopic eczema (Kerscher *et al.*, 1991). These studies indicate that ceramide can be used as an active component in dermatological therapy.

Synthetic ceramide is normally obtained by acylation of the amino group of a sphingosine, a sphinganine or their derivatives (Smeets and Weber, 1997; Semeria *et al.*, 1997; Cho *et al.*, 1995). Synthesizing optically pure sphingosine and its derivatives has been achieved by using serine as a chiral building block (Liotta and Merrill, 1992). Other approaches to synthesize isomerically pure sphingosine and its derivatives have employed other chiral starting materials, such as carbohydrates, L-glyceric and D-tartaric acids, etc. (Rochlin, 2002). However, all these synthetic approaches are tedious and time consuming for industrial applications. Moreover, the requirement of the proper stereochemistry of the end products limits further improvement of these synthetic processes. A recent patent uses D-galactose and benzaldehyde as starting material to prepare sphingosine and ceramide (Rochlin, 2002). This method is better, but still difficult for potential scale-up due to the many steps involved. Therefore, the development of alternative cost-efficient, high yield production methods is of great interest.

Sphingomyelin (SM), which contains a ceramide moiety, is a ubiquitous component of animal cell membranes. SM isolated from fish and shellfish (Takahashi *et al.*,

1995), as well as from erythrocytes (Hara *et al.*, 1999), has been hydrolyzed for ceramide production, with phospholipase C as a catalyst. This opens up a new strategy for the production of ceramide. Because SM is one of the major phospholipids in bovine milk, it is possible to obtain SM in large quantities. Another advantage of producing ceramide through the hydrolysis of the natural SM, is that the products will have the required stereochemistry. Since the ceramide in the natural SM has the same stereochemistry as the desired ceramide, isomeric purity of the final product from SM hydrolysis should be identical. Therefore, this approach for ceramide production has great potential for further applications at an industrial level, and should attract more interest. Unfortunately, only two brief reports (Takahashi *et al.*, 1995; Hara *et al.*, 1999) have focused on ceramide production using SM hydrolysis, and no systematic study has yet to be published. The main purpose of the present project was to systematically investigate and optimize the enzymatic production of ceramide from SM hydrolysis, as well as to develop an efficient and scalable process.

The modification site for producing ceramide from SM is a bond between the primary hydroxyl group of ceramide and choline phosphate ester. Since this bond cannot be broken specifically using chemical hydrolysis (Hara *et al.*, 1999), an enzyme-based method must be applied. Possible enzymes for catalyzing the hydrolysis reaction are sphingomyelinase (SMase, EC 3.1.4.12), and phospholipase C (PLC, EC 3.1.4.3). SMase is a specific enzyme for hydrolyzing SM. PLC is classified as a phosphoric acid diester hydrolase which catalyses the hydrolysis of glycerophospholipids to diacylglycerol and organic phosphate. Among various PLC, *C. perfringens* PLC is of particular interest, since it has been reported that this enzyme contains two different domains responsible for phosphatidylcholine and SM hydrolysis (Titball *et al.*, 1991).

1.1 Objectives and thesis outline

This thesis provided a better understanding on some important aspects concerning the enzymatic production of ceramide. The objectives of the present project were as follows:

- 1) Improvement of SM hydrolysis for ceramide production through system evaluation and optimization of the single-batch reaction.

- 2) Enhancement of the reusability of the enzyme through enzyme immobilization and optimization of SM hydrolysis catalyzed by the immobilized enzyme in the reaction system of interest.
- 3) Elucidation of possible reaction mechanisms and further improvement of SM hydrolysis through a kinetic study using both the soluble and immobilized enzyme.
- 4) Development of an enzyme membrane reactor for SM hydrolysis and evaluation of the application of the membrane reactor.
- 5) Comparison and assessment of various approaches developed for SM hydrolysis.
- 6) Evaluation of the possibility to synthesize more structurally-complicated ceramide through other enzymatic methods than SM hydrolysis.

The present thesis was divided into three main parts.

The first part consists of the introduction and the background of this study (Chapter 1-3). Chapter 1 summarizes the background, describes the objectives of the study, and introduces the structure of the thesis. Chapter 2 presents natural sources, structure, properties and functions of ceramide and SM. The current methods for ceramide production and sources of SM are also presented. Chapter 3 discusses the enzymes and reaction systems related to the enzymatic production of ceramide. The classification, function and characteristic of the enzymes are introduced. The reaction system for SM hydrolysis is reviewed. Furthermore, potential methods to improve SM hydrolysis, enzyme immobilization and the application of enzyme membrane reactors are also discussed.

The second part of the thesis describes the experimental work, the results and the conclusions (Chapter 4-6). Experimental work and analytical approach is given in Chapter 4. The results and their discussion are summarized in Chapter 5. Based on the results, conclusions were made in Chapter 6, where the future perspectives are also discussed.

Finally the third part contains the four scientific articles published together with three appendices, which are not included in the articles. The details of the experimental approach, results and discussion are described individually in the articles and the appendices.

2 Background of ceramide and sphingomyelin

2.1 Ceramide: structure, properties and functions

Ceramide consists of a long-chain sphingosine base linked to a fatty acid via an amide bond (Fig. 1). Natural ceramides have D-erythro configuration, and are structurally heterogeneous. The number and the position of the hydroxide group and the saturation level vary in the sphingosines in natural ceramides. The fatty acids in natural ceramides contain from 12 to 28 carbon atoms, either saturated or monosaturated, and in some cases contain a hydroxyl group at either the C'2 (α position) or terminal carbon atom (ω position) (Goñi and Alonso, 2006). Ceramide is the key intermediate in the biosynthesis of all the complex sphingolipids (Fig. 2).

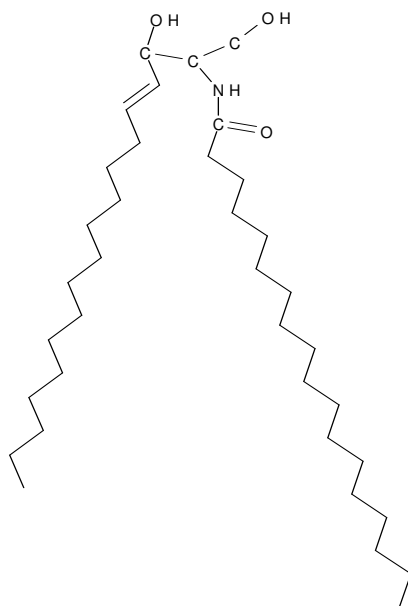


Figure 1: The structure of ceramide.

Ceramide has a multitude of functions in cellular physiology; hence it has attracted much attention in the last two decades. In the cell membrane, ceramide shows a strong tendency to form highly ordered ceramide-enriched microdomains, which facilitate signal transduction initiated by many stimuli (Bollinger *et al.*, 2005). Ceramide has an important role in cellular signaling, and especially in the regulation of apoptosis, cell differentiation, transformation and proliferation (Lahiri and Futerman, 2007;

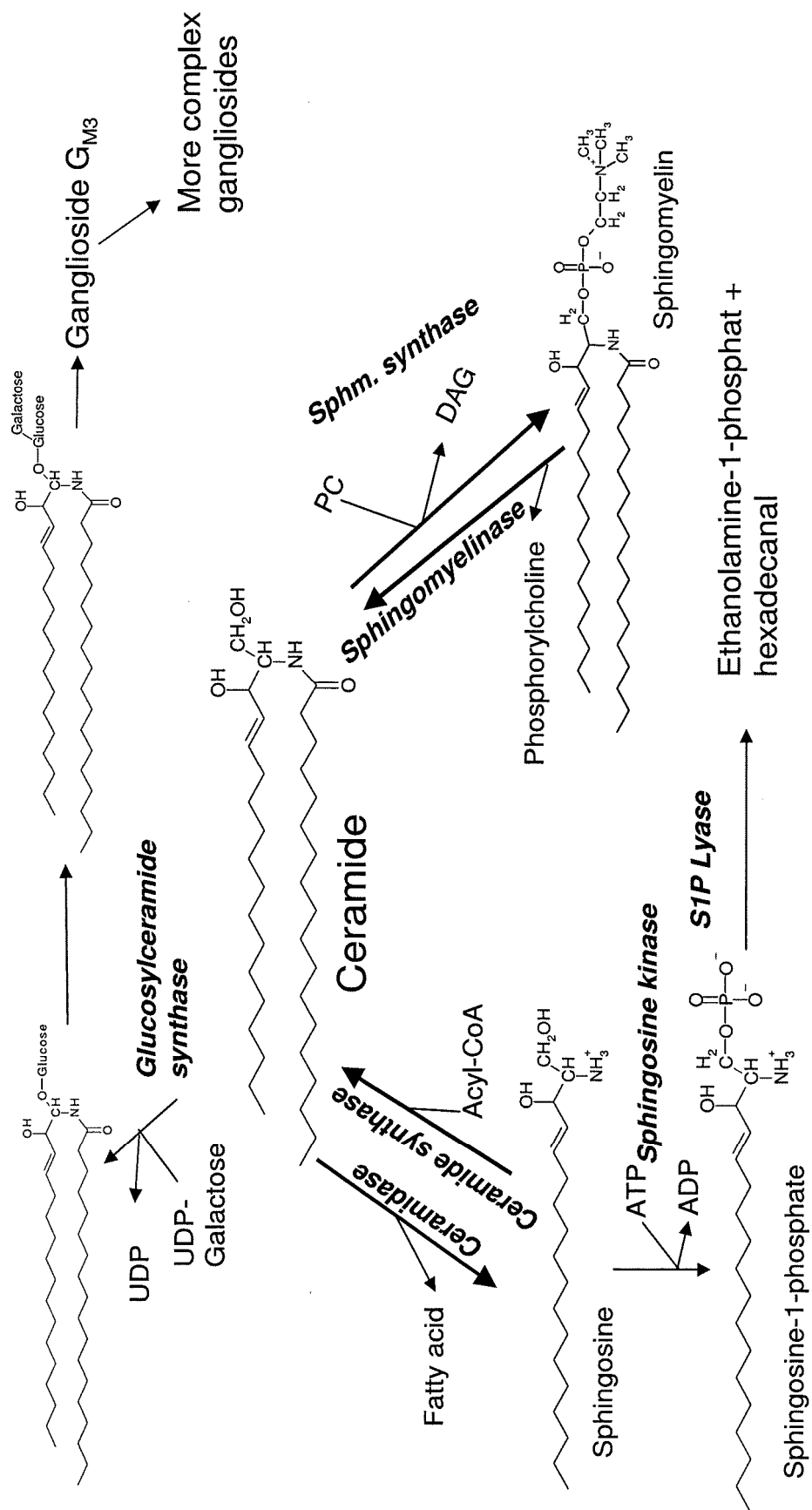


Figure 2: Sphingolipid metabolism (Hellgren LI, personal communication, 2008).

Chatterjee, 1999). It is a key component in intracellular stress response pathways (Hannun and Obeid, 2002).

Ceramide plays a major role in structuring and maintaining the water-retaining properties of the epidermis (Coderch *et al.*, 2003). Lipid layers present in the intercellular spaces of the stratum corneum of the skin form a "water barrier" which prevents water loss from the skin, a factor essential for survival (Coderch *et al.*, 2003). Ceramide, cholesterol and free fatty acids constitute the major lipid classes in the stratum corneum (Bouwstra and Ponec, 2006). In ceramides within human stratum corneum, sphingosine, phytosphingosine or 6-hydroxysphingosine are linked to a non-hydroxylated fatty acid or an α -hydroxy fatty acid of varying chain length (Bouwstra and Ponec, 2006). Eight ceramides have been identified from human stratum corneum, and they form a unique pattern, which plays an important role in organization and cohesion of skin structure (Coderch *et al.*, 2003). The total ceramide content decreases and the ceramide pattern is altered in most skin disorders that have a diminished barrier function (Coderch *et al.*, 2003). Healthy people with a low level of ceramide 1, ceramide 6I, and ceramide 6II are more susceptible to irritant contact dermatitis (Jungert *et al.*, 2008).

Ceramide has great commercial potential in cosmetics and pharmaceuticals, in for example, hair and skin care products. Application of 0.1 % ceramide in a hydrophilic ointment was found to have good healing effects on patients with atopic dermatitis (Hara *et al.*, 1999). Promising results were reported in a phase 1 trial of a ceramide-dominant, barrier repair moisturizer for childhood atopic dermatitis (Chamlin *et al.*, 2001). Ceramide-containing products are also effective in restoring the water content of dry skin and in relieving atopic eczema (Kerscher *et al.*, 1991). These studies indicate that ceramide can be used as an active component in dermatological therapy. Many products containing ceramide or its synthetic analogues have already been introduced in the cosmetics market, with excellent results. A cosmetics company (Parfums Raffy Inc., California, U.S.A) claim that these products dramatically increase the skin's hydration level, repair the cutaneous barrier, prevent vital moisture loss, and contribute to reducing dry flaky skin and an aged appearance.

The important role of ceramide in cellular signaling raises the issue of whether the presence of ceramide in the stratum corneum impacts the underlying epidermis, resulting in proliferation, differentiation, etc. Since ceramides in the stratum corneum have a fatty acyl chain length of at least 24 carbon atoms, they are so hydrophobic that free ceramides cannot exist in biological fluids or in cytosol (Goñi and Alonso, 2006). Due to this extremely hydrophobic nature, it is difficult for ceramide to penetrate through the intact stratum corneum to influence the underlying epidermis (Harding *et al.*, 2000). Therefore, the effect of ceramide from the stratum corneum on the underlying epidermis is negligible.

2.2 Ceramide production

2.2.1: Conventional methods

Ceramide is primarily obtained via extraction and isolation from animal sources, such as bovine brain, porcine epidermal tissues, eggs, blood cells, etc. (Smeets and Weber, 1997; Smeets *et al.*, 1997; Semeria *et al.*, 1997). These extracts generally contain only a few percent ceramide (Smeets and Weber, 1997). On a large scale, the production of ceramide using these methods is rather costly. Since these mixtures are subject to bacterial contamination, their preservation is difficult to control. In addition, these materials are potentially risky owing to the potential for contamination by the agent responsible for bovine spongiform encephalopathy (BSE) (Smeets *et al.*, 1997; Semeria *et al.*, 1997).

In order to overcome these problems, several chemical methods for the synthesis of ceramide have been proposed (Smeets and Weber, 1997; Rochlin, 2002; Semeria *et al.*, 1997; Cho *et al.*, 1995). It has already been reported that certain compositions of synthetic ceramide in have exhibited moisturizing effects similar to natural ceramide (Bouwstra and Ponec, 2006). Synthetic ceramide is normally obtained through the acylation of the amino group of sphingosine, sphinganine or their derivatives (Smeets and Weber, 1997; Semeria *et al.*, 1997; Cho *et al.*, 1995). Synthesizing optically pure sphingosine and its derivatives was achieved by using serine as a chiral building block (Liotta and Merrill, 1992). Other approaches to synthesize isomerically pure sphingosine and its derivatives have employed other chiral starting materials, such as

carbohydrates, L-glyceric and D-tartaric acids, etc. (Rochlin, 2002). However, all these synthetic approaches are tedious and time consuming for industrial applications. A recent patent reportedly used D-galactose and benzaldehyde as starting material to prepare sphingosine and ceramide (Rochlin, 2002). This method is better, but still difficult for potentially scale-up due to the large number of steps involved.

Besides being laborious and expensive, chemical synthetic methods have other disadvantages. The requirement of the proper stereochemistry of the end products limits further improvement. Chemical synthetic methods may also result in undesired residual chemicals in the final products (Smeets *et al.*, 1997). With the many steps involved in chemical synthetic methods, large amount of by-products and waste are produced, and suitable treatment is needed to reduce their environmental impact. Moreover, large energy consumption means chemical synthetic methods for ceramide are not favorable for industrial applications.

2.2.2: Enzymatic and fermentation methods

Enzymes are protein catalysts that selectively lower the activation energies of chemical reactions. They are more efficient catalysts than any man-made catalysts yet devised (Price, *et al.*, 2001). They give both large reaction rate enhancements and a high degree of selectivity and specificity (Price, *et al.*, 2001). Because enzymes are able to catalyze reactions at mild conditions, enzymatic reactions normally require low energy consumption. Due to their unique properties, it would be advantageous to employ enzyme technology in the production of ceramide.

Enzymatic productions of ceramide have already been reported in the literature. Labelled ceramide has been synthesized from sphingosine and [^{14}C] fatty acid using sphingolipid ceramide *N*-deacylase (EC 3.5.1.23) from *Pseudomonas* sp. TK4 (Mitsutake *et al.*, 1997). Using lipase (EC 3.1.1.3) from *Pseudomonas alcaligenes*, ceramide and hybrid ceramide have been produced through the selective acylation of the amino group of sphingosine, dihydrosphingosine and phytosphingosine (4-hydroxydihydrosphingosine) (Smeets *et al.*, 1997). The products of the enzymatic reactions have the correct stereochemistry, and more closely resemble the natural

compounds compared to chemical synthetic methods. However, the starting materials employed in these methods are sphingosine or its derivatives, which are difficult to synthesize on a large scale as discussed above. Therefore, at present, further improvements to the method through decreases in cost are limited.

Ceramide containing phytosphingosine was produced through the combination of microbial fermentation and chemical methods (Casey *et al.*, 1998). The method is comprised of three steps: 1), tetraacetylphytosphingosine (TAPS) is produced by the fermentation of the F-60-10 mating type strain of *Hansenula ciferrii*; 2), phytosphingosine is obtained by the chemical deacetylation of TAPS; 3), phytosphingosine-containing ceramide is formed through the chemical acylation of the amino group of phytosphingosine with a fatty acid. Phytosphingosine can be produced in large quantities using this method. Therefore, ceramide production from phytosphingosine has a large advantage compared with previously mentioned production methods. However, most natural ceramides in the stratum corneum don't contain phytosphingosine, and they cannot be produced by this method. As well, in the fermentation medium, the microorganism often becomes inactivated by the product when the product reaches a certain critical concentration. As a result, the product is normally obtained only in a dilute form. Hence, it is necessary to provide additional steps for the concentration and purification of the product. Such steps are often difficult and expensive.

Currently, ceramide is not easy to produce for industrial applications. The price of synthetic ceramide is 1500-2000 € / kg, and ceramide with fatty acid compositions similar to those found in the skin costs several hundred thousand € / kg. Therefore, it is desirable to find an alternative cost-efficient and high-yield method for obtaining this valuable product. Ceramide production from sphingomyelin (SM) hydrolysis has been carried out using an enzyme, phospholipase C (PLC, EC 3.1.4.3), from the literature. To produce ceramide, SM isolated from the boiled juice of fish and shellfish (Takahashi *et al.*, 1995), as well as SM from erythrocytes (Hara *et al.*, 1999), were hydrolyzed with PLC as a catalyst. This provides a new strategy for the production of ceramide.

2.3 Structure and physiological function of sphingomyelin

Sphingomyelin is a ubiquitous component of animal-cell membranes. It is by far the most abundant sphingolipid. The structure of SM is described in Fig. 3. In SM, the ceramide part of the molecule is bound through a phosphodiester bridge to a choline moiety. The molecule has four possible stereoisomeric forms, and naturally occurring SM is D-erythro-SM. The natural SM is heterogeneous, differing in N-acyl chain length and saturation and the length of the sphingosyl-backbone (Ramstedt *et al.*, 1999; Table 1). In the external leaflet of cellular plasma membranes, SM's high packing density and affinity for cholesterol facilitate the formation of “rafts”, rigid sub-domains of membranes (Ramstedt and Slotte, 2002). It was suggested that these rafts take part in cellular processes, such as signal transduction, membrane trafficking and protein sorting (Ramstedt and Slotte, 2002).

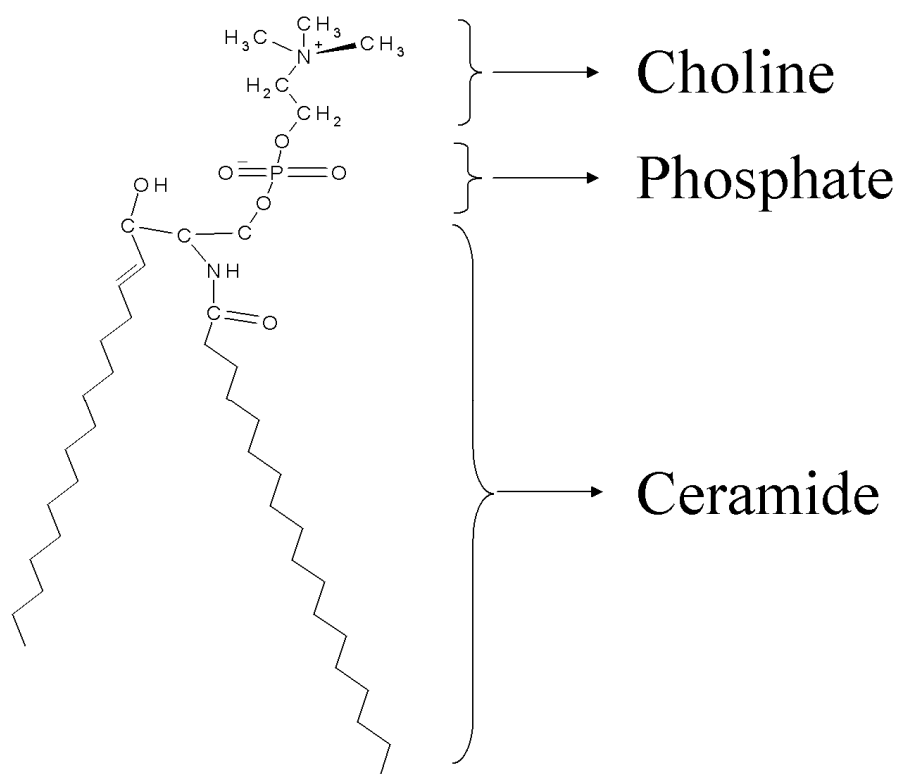


Figure 3: The structure of sphingomyelin.

Table 1: Fatty acid composition of SM (wt % of the total) in some animal tissues (Ramstedt *et al.*, 1999).

Fatty acids	16:0	18:0	18:1	20:0	22:0	22:1	23:0	23:1	24:0	24:1
Egg	66	10	1	4	6	1	2	-	5	3
Bovine brain	3	42	-	6	7	3	3	3	6	27
Bovine milk	14	3	1	1	22	-	32	-	19	5

As an essential membrane constituent, SM is also a metabolic source of lipid second messengers that regulate series of physiological events. In the sphingolipid metabolism (Fig. 2), SM serves as a precursor for ceramide, although ceramide can also be formed through de novo synthesis (Lahiri and Futerman, 2007).

2.4 Source of sphingomyelin

When evaluating the feasibility of ceramide production through SM hydrolysis, the availability of SM is an important factor and should be of primary concern. SM has been extracted from various sources, such as the bovine brain, chicken egg yolk, erythrocytes (Hara *et al.*, 1999) and sea products (Takahashi *et al.*, 1995). On the other hand, SM is also one of the major phospholipids in milk and most dairy products, with amounts equal to or higher than phosphatidylcholine (PC) (Liu *et al.*, 2000). Consequently, bovine milk is a safe and convenient source of SM compared to bovine brain tissue due to the potential spread of BSE (Bloomer, 2006). During the production of dairy products like cheese, waste containing abundant SM will be discarded. In a whey-fat phospholipid fraction (Lacprodan PL-75, Arla Foods Ingredients a.m.b.a, Viby J. Denmark), SM content was determined to around 25 %. A simple purification method, mild alkaline hydrolysis, was applied to purify SM from Lacprodan PL-75, and the purity of SM was increased from 25 % to 78 % with an 80 % recovery rate (Paper IV). Thus, dairy SM can be produced in large quantities, and is a potential substrate for ceramide production.

Another advantage of producing ceramide through the hydrolysis of natural SM, is that the product has the required stereochemistry. Since the ceramide in natural SM has the same stereochemistry as the desired ceramide, isomeric purity of the final product from SM hydrolysis is identical to the natural ceramide. Therefore, SM hydrolysis

may be a feasible and cost-effective method for the production of ceramide. Development of this production process and associated techniques is important for further applications on an industrial level, and should attract more attention. Unfortunately, only two brief reports (Takahashi *et al.*, 1995; Hara *et al.*, 1999) have focused on ceramide production through SM hydrolysis, and no systematic study has yet to be published. The main purpose of the present project was to systematically investigate and optimize the enzymatic production of ceramide from SM hydrolysis, as well as to develop an efficient and scalable process. Background relating to the enzyme and reaction system will be overviewed in the following chapter.

3 Enzyme and reaction system

The modification site for producing ceramide from SM hydrolysis is a bond between the primary hydroxyl group of ceramide and the choline phosphate ester (Fig. 3; Fig. 4). Since this bond cannot be broken specifically using a chemical hydrolysis (Hara *et al.*, 1999), an enzymatic method has to be applied. Fig. 4 shows the specific enzymes and their acting-sites for the modification of SM. Potential enzymes for catalyzing the hydrolysis reaction are sphingomyelinase (SMase, EC 3.1.4.12), and PLC. These two enzymes have different properties and will be introduced individually.

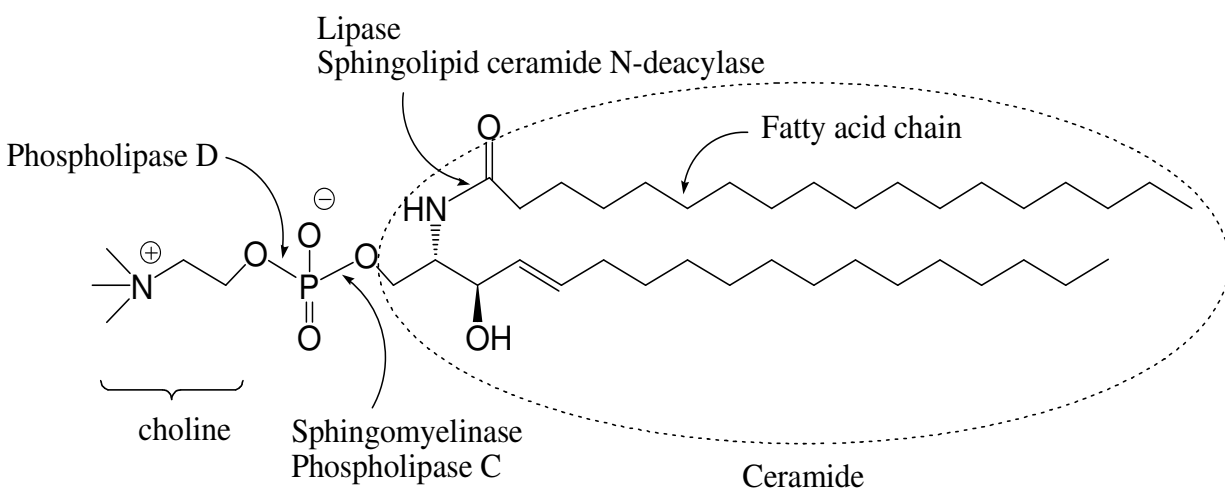


Figure 4: Structural moieties of sphingomyelin, specific enzymes and their acting-sites for the modification.

3.1 Sphingomyelinase

Sphingomyelinase is a specific enzyme capable of hydrolyzing SM into ceramide and phosphorylcholine. At least three types of SMase have been identified, classified according to their different optimal pH values. The optimal pH values of acid SMase, neutral SMase and alkaline SMase are approximately 4.5, 7.5 and 9.0, respectively (Liu *et al.*, 2000). Acid SMase is a lysosomal and secreted enzyme. Inherited deficiencies of acid SMase activity lead to different forms of Niemann-Pick disease, which are characterized by lysosomal SM accumulation (Kolzer *et al.*, 2004). Sources

of neutral SMase include biological fluids, solid tissues, cultured cells, or bacteria. The properties, isolations and biological functions of neutral SMase have been recently introduced in high-quality reviews (Wascholowski and Giannis, 2001; Chatterjee, 1999). Unlike acid SMase and neutral SMase, alkaline SMase plays an important role in SM digestion in the intestinal lumen. Hydrolysis of dietary SM in the intestinal tract is a slow and incomplete process, where phospholipids and their hydrolysis products influence the activity of alkaline SMase (Liu *et al.*, 2000).

The kinetic steps for the hydrolysis by SMase from *Bacillus cereus* have been studied in the monolayer reaction system (Fanani and Maggio, 2000). The reaction has a latency period before exhibiting a constant-rate hydrolysis. Enzyme-substrate binding and associated states with irreversible interfacial activation are presented before complete activation of the enzyme (Fanani and Maggio, 2000). The reaction catalyzed by the other important enzyme, PLC, also has a pre-catalytic activation step (Daniele *et al.*, 1996). These steps are assumed to be rate-limiting steps of the whole reaction.

3.2 Phospholipase C

In general, SMase is the most efficient enzyme for catalyzing the hydrolysis of SM. However, its price is so high (more than 4.5 € per U from the market) that its use on an industrial scale is not practical at present. A substitute enzyme to catalyze SM hydrolysis to produce ceramide is PLC, which is much cheaper than SMase. PLC is classified as a phosphoric acid diester hydrolase which catalyses the hydrolysis of glycerophospholipids to diacylglycerol (DAG) and organic phosphate. The substrate specificity of PLC has been found to vary depending upon both the source and type of PLC.

Although SM is not the preferred substrate of PLC, there is a connection between the hydrolysis of SM and the catalytic function of PLC. The structure of SM is similar to PC, which is one of the preferred substrates of PLC. Both of them contain a phosphorylcholine and an *sn*-2 carbonyl group, which are the common substrate features for binding to PLC from *Bacillus cereus* (Hansen *et al.*, 1993). Enzymatic SM hydrolysis to ceramide and phosphorylcholine has been achieved using several

preparations of PLC from bacteria, plants, and some animal sources (Barnholz *et al.*, 1966). PLC from two of the most common sources, *Clostridium perfringens* and *Bacillus cereus*, are presented in detail below.

PLC from *Clostridium perfringens* is lethal, necrotizing and hemolytic while most other bacterial PLC are entirely devoid of such activities (Takahashi *et al.*, 1981). The molecular weight for the purified enzyme (monomer) was determined to be 43,000 Daltons by SDS-polyacrylamide gel electrophoresis (Takahashi *et al.*, 1981). This PLC shows a broad specificity towards various phospholipids, and the rate of the hydrolysis by the enzyme decreases in the following order: PC > SM > phosphatidylserine (Takahashi *et al.*, 1981). With the catalysis by the highly purified enzyme, SM was hydrolyzed at 35% of the rate of PC and lysophosphatidylcholine was not significantly hydrolyzed (Krug and Kent, 1984). When SM and PC were adsorbed on the amphiphilic gel octyl-sepharose CL-4B by hydrophobic interaction, the rates of SM and PC hydrolysis catalysed by *Clostridium perfringens* PLC were similar (Malmqvist *et al.*, 1978). Therefore, the immobilization of the substrate affects the substrate specificity of the enzyme.

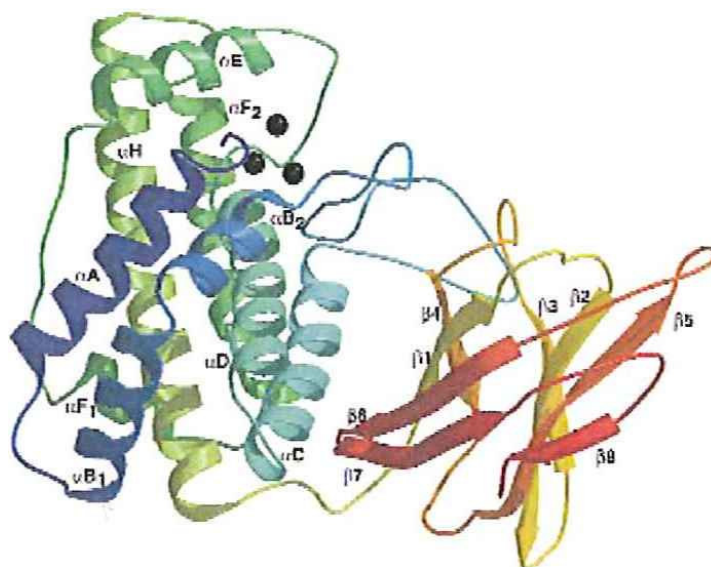


Figure 5: Structure of PLC from *Clostridium perfringens* (Naylor *et al.*, 1998). The protein sequence is shaded from blue at a residue 1 to red at a residue 370. Three black spheres indicate the position of zinc ions in the active site.

Elucidation of the crystal structure of PLC from *Clostridium perfringens* showed that the protein contained two domains: an α -helical amino-terminal (N) domain (residues 1-246) and a β -sheet-containing carboxy-terminal (C) domain (residues 256-370) (Fig. 5) (Clark *et al.*, 2003). The N-domain retains all the activity towards PC hydrolysis, but not the activity towards SM hydrolysis (Titball *et al.*, 1991). In the C-domain of the enzyme, the hydrophobicity of the 330-335 residue loop is important for membrane binding and residues Y331 and F334 play a vital role in the catalysis of SM hydrolysis (Clark *et al.*, 2003). It has been suggested that the C-domain facilitates PLC binding to the polar head groups of phospholipids, where their hydrolysis is catalyzed by the N-domain (Clark *et al.*, 2003).

Bacillus cereus secretes three phospholipases, the first (PI-PLC) specific for phosphatidylinositol hydrolysis, the second (SMase) specific for SM hydrolysis, and the third (PC-PLC) specific for the hydrolysis of PC, phosphatidylserine, and phosphatidylethanolamine (Waite, 1987). It has been found that PI-PLC does not catalyze SM hydrolysis (Ikezawa and Taguchi, 1981). PC-PLC is a simple monomeric protein containing two tightly bound Zn atoms and one loosely bound Zn atom (Fig. 6), and consists of 245 amino acids with a molecular weight of 28,520 Daltons (Epperson and Ming 2001; Hansen *et al.*, 1993). The metal ions play an important role in stabilizing the structure of the enzyme since they are coordinated to widely separated parts of the amino acid chain, and they are also directly involved in the catalytic process (Hansen *et al.*, 1993). Using a series of amino acid blocking agents, it has been found that two lysyl, one histidyl, one arginyl, and at least one carboxyl group are required for retaining the full activity (Little, 1981a; Waite, 1987).

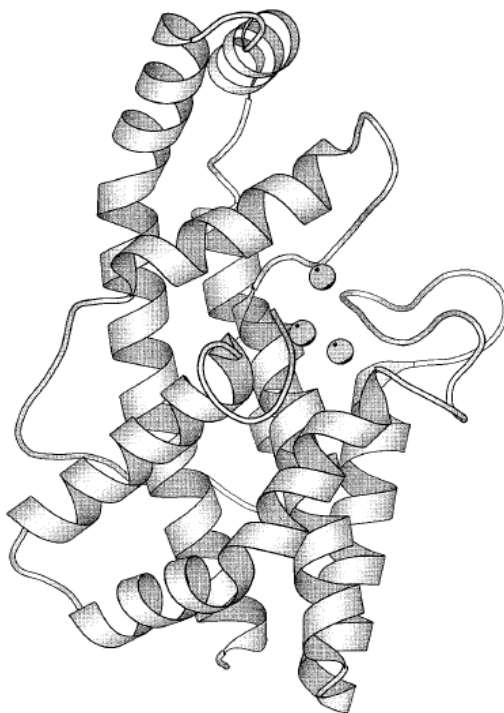


Figure 6: Three-dimensional representation of PC-PLC from *B. cereus* (Thrige *et al.*, 1997). The three zinc ions in the active site are shown as spheres.

Based on a fully energy-minimized structure of the enzyme–substrate complex, a study has proposed a PC-PLC catalytic mechanism, involving two steps (Thrige *et al.*, 1997): Firstly, a nucleophilic water molecule activated by an acidic residue (Asp55) attacks the phosphate group of the substrate to generate a trigonal bipyramidal pentacoordinated intermediate; Secondly, the intermediate collapses to release the hydrolysis products and to regenerate the enzyme.

When in deoxycholate-mixed micelles, PC, phosphatidylserine, phosphatidylethanolamine but SM were readily hydrolyzed with the catalysis by *Bacillus cereus* PC-PLC (Little, 1981a). However, electrophoretically pure recrystallized PC-PLC catalyzed the hydrolysis of SM both in the myelin sheath and in the membrane of human red cells (Little, 1981a; Little *et al.*, 1982). The same enzyme preparations are about 2000-fold more active with pure PC-deoxycholate micelles than with SM-deoxycholate micelles (Little, 1981a). Another study indicates that the highly purified PLC can catalyze SM hydrolysis, although the hydrolysis rate is no more than 1 % of the rate in PC hydrolysis (Waite, 1987).

Some approaches to change the substrate specificity of PC-PLC from *Bacillus cereus* have enhanced SM hydrolysis reaction. The metal content of the enzyme influences the state of the enzyme and affects its substrate specificity. *O*-phenanthroline is a chelating agent, which rapidly removes the metal atoms from the enzyme. When PC-PLC was inactivated by *o*-phenanthroline and reactivated by Co^{2+} , the rate of SM hydrolysis was increased to approximately 30 % of the rate in PC hydrolysis (Otnaess, 1980). In another study, when the total metal content was changed to Co^{2+} by adding Co^{2+} to the apoenzyme of PC-PLC, the activity towards SM hydrolysis was 4-7 fold greater than that of the native enzyme containing only Zn^{2+} (Little, 1981b). When the dialysis of the native enzyme against *o*-phenanthroline was used to control the content of Zn^{2+} , the enzyme containing one mol Zn^{2+} /mol of the enzyme had the enhanced activity towards SM hydrolysis, although near Zn^{2+} -free enzymes produced by longer exposure to *o*-phenanthroline had less activity towards SM hydrolysis (Little, 1981b). Therefore, the exact influence of the metal content on the activity towards SM hydrolysis is not clear, and further investigations are required.

^{31}P nuclear magnetic resonance studies showed that inactivated PC-PLC from *Bacillus cereus* could bind SM, and catalyze its hydrolysis (Aalmo *et al.*, 1984). PC-PLC was 99.9 % inactivated (towards PC hydrolysis) by pretreatment with the histidine reagent diethylpyrocarbonate, a process that inactivated the enzyme without destroying its ability to bind substrate (Aalmo *et al.*, 1984). Bulk association between PC-PLC and SM followed by SM hydrolysis was detected in the study, indicating that the activity towards SM hydrolysis is an inherent property of this enzyme rather than arising from trace contamination by another enzyme.

In my previous master project, both *C. perfringens* PLC and *B. cereus* PLC were pretreated with the histidine reagent or subject to exchange of metal ion content in the enzyme, using previously studied methods (Aalmo *et al.*, 1984; Otnaess, 1980). All the enzymes were inactivated by the pretreatment, and no activity towards SM hydrolysis was detected. The enzymes used were from commercial sources, while in the other studies (Aalmo *et al.*, 1984; Otnaess, 1980), the enzymes were prepared directly by the authors. Therefore, the different preparations of the enzymes contributed to the discrepancy between the results. In addition, differences in the

reaction system, the analysis method and the sensitivity of the assay between the master project studies and the reference could have also lead to discrepancies between the results.

3.3 Overview of current reaction systems

How the substrate is presented to the enzymes is critical in determining the rate of the hydrolysis catalyzed by PLC (Jungner *et al.*, 1997; Little, 1977a; Little, 1981b; Little *et al.*, 1982). Since SM is a water-insoluble lipid, the enzymatic reaction can only be performed in an organic solvent-water system, a monolayer system or a micelle-in-water system, that needs a detergent to prevent substrate aggregation at high SM concentrations. Table 2 shows an overview of previous research on SM hydrolysis using various enzymes and reaction systems. It should be mentioned that most of the studies listed in the table focus on physiology and enzymology, rather than the production of ceramide. Nonetheless, their evaluation is essential when designing a reaction system specific to the production of ceramide.

Table 2: Literature studies on the enzymatic hydrolysis of SM.

Enzyme	Reaction system	Reference
SMase from rat brain	Micelle-in-water with triton X-100	Gatt <i>et al.</i> , 1974
PLC encoded by a <i>P. falciparum</i> gene	Micelle-in-water with triton X-100	Hanada <i>et al.</i> , 2002
PLC from <i>C. perfringens</i>	Micelle-in-water	Pastan <i>et al.</i> , 1968
PLC from <i>B. cereus</i>	Micelle-in-water	Otnaess, 1980
SMase form rat brain	Micelle-in-water with triton X-100 or sodium cholate	Barnholz <i>et al.</i> , 1966
PLC from <i>A. saitoi</i>	Micelle-in-water with triton X-100	Matsuoka <i>et al.</i> , 1987
SMase from rat liver	Micelle-in-water with sodium cholate	Kanfer <i>et al.</i> , 1966
Rat intestinal alkaline SMase	Micelle-in-water with taurocholate	Liu <i>et al.</i> , 2000

PLC from <i>B. cereus</i>	Micelle-in-water with sodium deoxycholate	Little, 1981b
PLC from <i>B. cereus</i>	Micelle-in-water with sodium deoxycholate	Aalmo <i>et al.</i> , 1984
SMase from <i>B. cereus</i>	Monolayer	Fanani and Maggio, 2000
SMase from <i>S. aureus</i>	Monolayer	Ramstedt and Slotte, 1999
SMase from <i>S. aureus</i>	Monolayer	Jungner <i>et al.</i> , 1997
Crude enzyme from <i>B. cereus</i>	Two-phase system (diethylether: water, 50:50) and packed bed reactor (the mobile phase is water-saturated diethylether)	Hara <i>et al.</i> , 1999
PLC from <i>C. perfringens</i>	Two-phase system (diethylether: water) and packed bed reactor (the mobile phase is diethylether)	Takahashi <i>et al.</i> , 1995
PLC from <i>B. cereus</i>	Erythrocyte ghosts or myelin were suspended in water	Little <i>et al.</i> , 1982; Aakre and Little, 1982
PLC from <i>C. perfringens</i>	Dispersion of SM in ethanol with sodium deoxycholate	Krug and Kent, 1984

As seen in Table 2, micelle-in-water systems have been broadly applied for SM hydrolysis, together with the use of detergents, triton X-100 (Gatt *et al.*, 1974), sodium cholate (Kanfer *et al.*, 1966) or sodium deoxycholate (Aalmo *et al.*, 1984). Micelles are considered as superior substrates for catalysis by PLC from *Bacillus cereus*, because of significant increases in the rate of hydrolysis with the substrate concentration above the critical micelle concentration (Little, 1977a). Since the long acyl chains of natural phospholipids will protrude well beyond the surface of the enzyme, it is easy for PLC to bind the substrate and catalyze the hydrolysis in the hydrophobic environment within the micelle (Hansen *et al.*, 1993). The addition of detergent to a micelle-in-water system stimulated the catalytic activity of alkaline SMase (Liu *et al.*, 2000). In PC hydrolysis, the presence of detergents can also enhance the activity of both *B. cereus* PLC and *C. perfringens* PLC (Little, 1977a;

Takahashi *et al.*, 1981). It has been suggested that this stimulation of PC hydrolysis is caused by solubilization of DAG which accelerates the product release (Elsayed and Roberts, 1985). When synthetic PC was immobilized on activated agarose matrices, the addition of 1 % triton X-100 to the reaction mixtures did not affect the activity of *B. cereus* PLC (Delfino *et al.*, 1994). This result indicates that the immobilization of the substrate can remove the detergent effect on the activity of the enzyme.

However, the micelle-in-water system is unsuitable for the large scale production since issues such as separation of product from the reaction mixture and the requirement of high-speed stirring to prevent aggregate formation complicate the practical application of the procedure. Ceramide is insoluble in water. The purification of ceramide from the reaction mixture normally involves organic solvent extraction, which increases the number of process steps. In other reactions catalyzed by lipase, the productivity of the micelle-in-water system is relatively low (Balcao *et al.*, 1996). Furthermore, inclusion of detergents in the system could also interfere with the product analysis and also make the product separation more difficult.

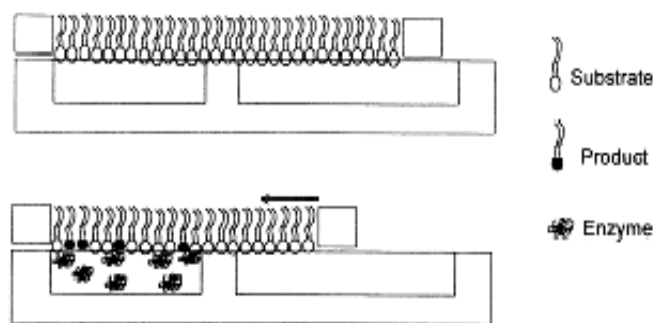


Figure 7: A simplified schematic view of the monolayer system using trough and compartments (Fanani and Maggio, 2000)

A monolayer system, as shown in Fig. 7, has been used in SM hydrolysis. In the system, lipid monolayers were spread from premixed solutions of the desired lipid in chloroform-methanol over a water phase containing the enzyme (Fanani and Maggio, 2000). Since the molecular area of ceramide is smaller than that of SM, the monolayer area will be decreased by SM hydrolysis at constant surface pressure. Therefore, the hydrolysis reaction could be detected on line from the change of monolayer area at constant surface pressure (Jungner *et al.*, 1997; Ramstedt and Slotte, 1999). This will

simplify the operation and make the kinetic research more precise. However, due to the need of larger operation area, a monolayer system is not quite feasible for ceramide production in industrial applications.

As seen in Table 2, a two-phase (diethylether: water) system was introduced in SM hydrolysis (Takahashi *et al.*, 1995; Hara *et al.*, 1999), whereby SM was dissolved in the organic solvent phase, and PLC was dissolved in the water phase. The hydrolysis was postulated to occur at the interface, where the enzyme and the substrate could interact. The product, ceramide, remained in the organic solvent phase. This makes product separation much easier than for a micelle-in-water system. Nevertheless, these two patents gave only brief information about the reaction. Many important factors, such as temperature, pH, organic solvent amount, etc. could have influenced the hydrolysis reaction; however, they were not investigated or evaluated in these reports. Moreover, the organic solvent used was diethylether, which is a toxic and extremely flammable material. The use of diethylether in large amounts is difficult, and requires much caution to avoid explosions. Consequently, the reaction system of SM hydrolysis reported in the literature is not suitable for industrial applications, and more systematic studies are essential to establish an enzymatic method for the production of ceramide.

3.4 Immobilization of enzyme

Enzyme immobilization is a process that involves attaching the enzyme onto an insoluble solid support with its retaining catalytic activity. Through immobilization, the enzyme can be physically separated from the bulk reaction medium and, at the same time, permeable to reactant and product molecules (Balcao *et al.*, 1996). The advantages of enzyme immobilization are:

1. The enzyme can be reused and utilized in a more economical way.
2. Enzyme stability is usually increased through the immobilization.
3. Immobilized enzymes often offer better processing possibilities than soluble enzymes.
4. Enzyme immobilization avoids the contamination of the products by residual enzymatic activity.

5. In organic media, immobilized enzymes often have higher observed catalytic activity than nonimmobilized enzyme powder because immobilization spreads enzymes on a relatively large area (Adlercreutz, 2006).

There are four general approaches for achieving enzyme immobilization (Holme and Peck, 1998; Fig. 8):

1. Adsorption: the enzyme is non-covalently adsorbed to an insoluble particle.
2. Covalent binding: the enzyme is covalently attached to an insoluble particle.
3. Entrapment: the enzyme is entrapped within an insoluble particle by a cross-linked polymer.
4. Encapsulation: the enzyme is confined within a semi-permeable membrane.

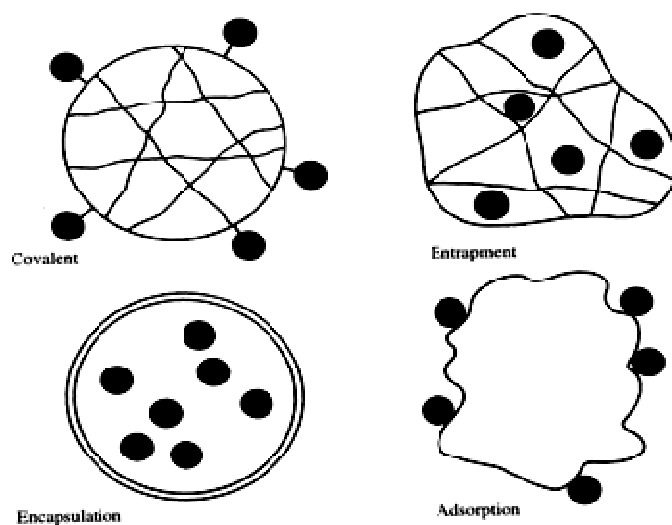


Figure 8: General methods used in enzyme immobilization (Holme and Peck, 1998). Black spheres indicate enzyme molecules.

Currently, the price of PLC is relatively high for industrial applications. This hinders the further development of large scale ceramide production. Thus, it is necessary to consider the reuse of the enzyme, which would significantly reduce the enzyme cost. Hundreds of studies in the area of enzyme immobilization have focused on the application of lipase, which has been summarized in some good reviews (Balcao and Malcata, 1998; Balcao *et al.*, 1996; Villeneuve *et al.*, 2000). However, the immobilization of PLC for the improvement of SM hydrolysis has not been emphasized or systematically studied. Table 3 is a literature survey of immobilization studies employing phospholipases.

Table 3: A literature survey on the immobilization of phospholipases (abbreviation: PLA₂, Phospholipase A₂; PLD, Phospholipase D)

Enzyme	Substrate	Method of immobilization	Support for immobilization	Retention activity ^a	Stability	Ref
Bee venom PLA ₂	Soybean PC	Adsorption (ion exchange)	Carboxymethyl Sephadex C-25	82 %	Full activity after 8 cycles	Madoery <i>et al.</i> , 1995
Cobra venom PLA ₂	Egg PC	Covalent binding	Acrylic polymer Eupergit C	48 %	Full activity after 10 cycles	Madoery and Fidelio, 2001
Cobra venom PLA ₂	PC	Covalent binding	Two kinds of agarose beads	> 50 %	NA	Ferreira <i>et al.</i> , 1993
Acylated snake venom PLA ₂	synthetic PC	Covalent binding	Cross-linked agarose beads	25 fold	NA	Shen and Cho, 1995
Snake venom PLA ₂	Phospholipid analog	Entrapment	A silica sol-gel matrix	NA ^b	Activity remains after several cycles	Fuentes <i>et al.</i> , 2001
Porcine pancreas PLA ₂	Egg yolk PC	Entrapment	Alginate-silicate sol-gel matrix	NA	Activity remains after 250 h operation	Kim <i>et al.</i> , 2001
Porcine pancreas PLA ₂	Soybean PC	Adsorption	7 carriers were tested. Amberlite XAD7 was suitable	NA	NA	Vikbjerg <i>et al.</i> , 2007
Bovine PLA ₂	Egg & fluorescent Phospholipids	Covalent binding	controlled pore glass beads	Nearly 100 %	Activity remains over 3 weeks storage	Nam and Walsh, 2004

<i>Pseudomonas</i> PLD	Soybean lecithin	Adsorption	Calcium alginate gel-enveloped polyethylene-glutaraldehyde	NA	Most activity remains after 15 batches	Wang <i>et al.</i> , 1997
Cabbage PLD	Ovolecithin	Adsorption	Polymeric carriers containing long chain anchor groups	0-28.4 %	Activity decrease a little after 5 batches	Lambrech and Ulbrichhofmann, 1993
<i>Streptomyces</i> PLD	Soybean PC	Covalent binding	Activated aminopropyl-glass and VA-Epoxy Biosynth	3.02-12.6 %	Activity remains after 3 months storage	Dittrich and Hofmann, 2001
<i>Streptomyces</i> PLD	PC and phosphatidylserine	Adsorption	Dowex MSA-2 macroporous resin	> 42%	80 % activity remains for 16 d operation	Yon <i>et al.</i> , 2008
<i>B. cereus</i> PLC	Soybean PC	Adsorption and covalent binding	Amberlite XAD7, Sepabeads FP- DA, Eupergit C, Celite 547, Silica gel 60	At most 21%	Activity remains after 6 cycles (using Sepabeads)	Anthonsen <i>et al.</i> , 1999
<i>C. perfringens</i> PLC	SM from fish, shellfish	Covalent binding	Amberlite XAD-8	NA	NA	Takahashi <i>et al.</i> , 1995
Crude enzyme from <i>B. cereus</i>	SM from erythrocytes	Covalent binding	Amberlite XAD-8	NA	NA	Hara <i>et al.</i> , 1999

a. Specific activity of the immobilized enzyme compared with the soluble enzyme

b. NA: Not available

The observed properties of the enzyme are invariably changed by immobilization. Studies in Table 3 illustrate that the operational and storage stability of the enzymes are dramatically increased by immobilization, confirming the advantage of enzyme immobilization. The retention of activity after immobilization is unpredictable, varying from zero to 25 fold compared with the soluble enzyme, with activity dependant on choice of immobilization method, type of enzyme preparation, support materials, etc.. Covalent binding is the most common method for the immobilization of phospholipases. Since covalent attachment between the enzyme and carrier is very strong, this type of immobilization method is more stable than others. Adsorption is a simple, less expensive and less labor-intensive method for immobilization. Because of the relatively weak interactions between the enzyme and the carrier, immobilization by adsorption will only have a minor effect on the activity of the enzyme (Vikbjerg *et al.*, 2007). Support materials used in immobilization studies are quite diverse; therefore, screening of suitable carriers made up of different support materials is necessary in future studies of PLC immobilization, in order to facilitate ceramide production on a large scale.

3.5 Enzyme membrane reactor

In the last two decades, many studies on enzyme membrane reactors in lipid technology have been carried out (Table 4). The enzyme membrane reactor refers to both the reactor that includes an enzyme-immobilized membrane, and the reactor where enzyme is suspended in solution and compartmentalized by a non-catalytic membrane (Sirkar *et al.*, 1999; Giorno and Drioli, 2000). In the present thesis, enzyme membrane reactors refer to the first type.

Due to both the intrinsic characteristics of the reaction system and enzyme properties, the enzyme membrane reactor offers a good chance to improve reaction performance. In lipid technology, the use of organic solvent or an aqueous-organic biphasic solvent system as reaction medium is very common. This promotes the application of the enzyme membrane reactor, based on two reasons: Firstly, when an anhydrous organic solvent is used as the reaction medium, the maintenance of an appropriate hydrophilic microenvironment around the enzyme structure results in a clear improvement in the stability (Klibanov, 2001). As such, a membrane composed of hydrophilic materials may be used in enzyme membrane reactors to provide an adequate hydrophilic

microenvironment or to retain an adequate water content in the anhydrous organic media (Lozano *et al.*, 2002). Secondly, many lipases and phospholipases have a high catalytic activity at the interface between the organic and water phases. In an aqueous-organic biphasic solvent system, an enzyme-immobilized microporous/porous membrane can be used to separate the two liquid phases, with their interface fixed at the membrane (Sirkar *et al.*, 1999). This can make the interface stable and easy to control. Consequently, the membrane at the interface will provide a large interfacial area for the immobilized enzyme to interact with the substrate, and enhance catalytic efficiency.

Besides these specific advantages related to lipid technology, application of the enzyme membrane reactor has other benefits (Sirkar *et al.*, 1999; Mulder, 1996a):

1. In an enzyme membrane reactor, the separation of products from the reaction mixture can potentially prevent product inhibition and side reactions. Therefore, the yield and productivity could be increased.
2. Regarding product separation, the enzyme membrane reactor is able to combine bioconversion and product purification steps together, thereby reducing the number of processing steps and operational costs.
3. Quite often, the stabilities of the enzyme are improved by immobilization in /on the membrane.

Table 4. A literature survey of enzyme membrane reactors employed in the area of lipid technology

Enzyme	Membrane material	Immobilization process	Remarks	Reference
<i>Candida antarctica</i> lipase B	α -alumina tubular membrane coated with hydrophilic inert polymers	Covalent binding (with glutaraldehyde)	Excellent stability	Lozano <i>et al.</i> , 2004
<i>Candida antarctica</i> lipase B	α -alumina tubular membrane coated with hydrophilic polymers	Covalent binding (with glutaraldehyde)	Good storage stability	Magnan <i>et al.</i> , 2004
<i>Candida rugosa</i> lipase	Regenerated cellulose (C030F), polyethersulphone (PM30)	Adsorption, inclusion by filtration and covalent binding	Inclusion by filtration show best activity	Hilal <i>et al.</i> , 2006
<i>Streptomyces</i> PLD	Polyacrylamide (Sepracor MBR-500™)	Ultrafiltration	Low yield, high stability	D'Arrigo <i>et al.</i> , 2005

<i>Candida rugosa</i> lipase	Polypropylene, regenerated cellulose and meta-aramid	Adsorption and filtration	Surfactant induced breakthrough	Vaidya <i>et al.</i> , 1994
<i>Candida antarctica</i> & pancreatic lipase	Polypropylene, nitrocellulose, cellulose and polyamide	Adsorption, inclusion by packing and covalent binding	Adsorption and packing show high activity	Trusek-Holownia and Noworyta, 2002
<i>Rhizopus javanicus</i> lipase	Polypropylene	Filtration	Hydrophilic membrane is better	Bouwer <i>et al.</i> , 1997
<i>Candida rugosa</i> lipase	Cellulose, cellulose derivatives and cellulose composite	Covalent binding (sodium periodate, carbodiimide)	Activity is the same order as literature	Carneiro-da-Cunha <i>et al.</i> , 1999
<i>Mucor miehei</i> lipase	Nylon, nylon coated with PVA	Covalent binding (glutaraldehyde)	Coating raise stability	Bruno, <i>et al.</i> , 2004
<i>Candida rugosa</i> lipase	pHEMA-MAPA membranes	Covalent binding (carbodiimide)	High stability	Bayramoglu <i>et al.</i> , 2002
<i>Candida rugosa</i> lipase	Polypropylene hollow fiber microfiltration membrane	Adsorption	High stability	Deng <i>et al.</i> , 2005
<i>Candida rugosa</i> lipase	Chitosan-tethered PANCMA hollow fiber membrane	Covalent binding (glutaraldehyde, EDC/NHS)	Low activity, high stability	Ye <i>et al.</i> , 2005
<i>Candida cylindracea</i> Lipase	Capillary tubes of aromatic polyamide	Covalent binding (glutaraldehyde)	Activity & stability in biphasic system are high	Molinari <i>et al.</i> , 1994

Commonly-used membrane materials include polypropylene (most common), cellulose, polyamide and ceramic (Table 4). Polypropylene is an excellent solvent resistant polymer that shows good chemical and thermal stability (Mulder, 1996b). Since it has a hydrophobic nature, it can be applied to maintain the organic solvent phase when the membrane is fixed at the interface in a biphasic reaction system. Cellulose and its derivatives are hydrophilic polymers, and also have outstanding membrane properties. Because they contain hydroxyl groups, they can covalently bind with the enzyme following activation by a certain agent (Carneiro-da-Cunha *et al.*, 1999). It is necessary to point out that some studies have focused on complex membrane materials e.g. a hydrophobic membrane combining (or coating) with a hydrophilic layer, or a hydrophobic layer incorporated into a hydrophilic membrane matrix (Table 4). In most cases, this special arrangement not only confers high efficiency during the immobilization process, but also improves the stability of the

enzyme-immobilized membrane (Table 4). Therefore, complex membrane materials will be used more in future.

Of the various types of immobilization methods available, covalent binding, which gives the strongest connection between the enzyme and the membrane, is most commonly used (Table 4). However, this method normally requires activation reagents, such as glutaraldehyde (Lozano *et al.*, 2004), sodium periodate (Carneiro-da-Cunha *et al.*, 1999) or carbodiimide (Bayramoglu *et al.*, 2002). Some enzymes may be denatured by this method, or have their active sites blocked during the immobilization process. These are the main reasons why enzymatic activities may not be retained; however, other immobilization methods may still result in even higher activities (Hilal *et al.*, 2006; Trusek-Holownia and Noworyta, 2002). Adsorption and filtration are also commonly used as immobilization methods in the lipid area (Table 4). These methods are simple and convenient. In addition, they often give higher activity retention than covalent binding. However, since the bond between the enzyme and the membrane is not as strong using these methods, the enzyme may be easily released from the membrane after a certain period of time. Hence, there is no set rule for selection of an immobilization method; it is recommendable to try several immobilization methods to find the best one for specific cases.

In the area of lipid technology, the specific activity of the immobilized enzyme is normally low compared with the soluble enzyme, though one exception has been published (Ye *et al.*, 2005), where the immobilized enzyme retained 45 % of the activity of the soluble enzyme. On the other hand, enzyme stability usually improves following immobilization in an enzyme membrane reactor (Table 4). The enzymes are normally expensive for industrial applications. Therefore, if the enzymes can be recycled, operated or stored for a prolonged length of time with little loss of their activities, industry can save on cost. In summary, lots of studies have already illustrated the benefits and the potential of enzyme membrane reactors. Consequently, applications using enzyme membrane reactors in the lipid area will likely get more and more attention in future. However, no literature is currently available addressing an enzyme membrane reactor for SM hydrolysis.

4 Experimental approach

The present project is a technical and engineering study on the enzymatic production of ceramide. Fig. 9 is an overview of the experimental approach, detailing related papers and appendices. As shown in the overview, the first and main part of this thesis focuses on SM hydrolysis for the production of ceramide. In this part, the enzymatic production of ceramide from SM hydrolysis was systematically investigated and optimized. Enzyme immobilization and the enzyme membrane reactor, which have the potential to improve the efficiency and feasibility of the reaction system, had been developed for industrial applications. Another part of this thesis studied the enzymatic synthesis of more structurally-complicated ceramides. Several schemes for the synthesis have been proposed, and the enzymatic acylation of sphingosine has been investigated in this part. Besides these two parts, the methods for data analysis and sample analysis have also been introduced in the present chapter.

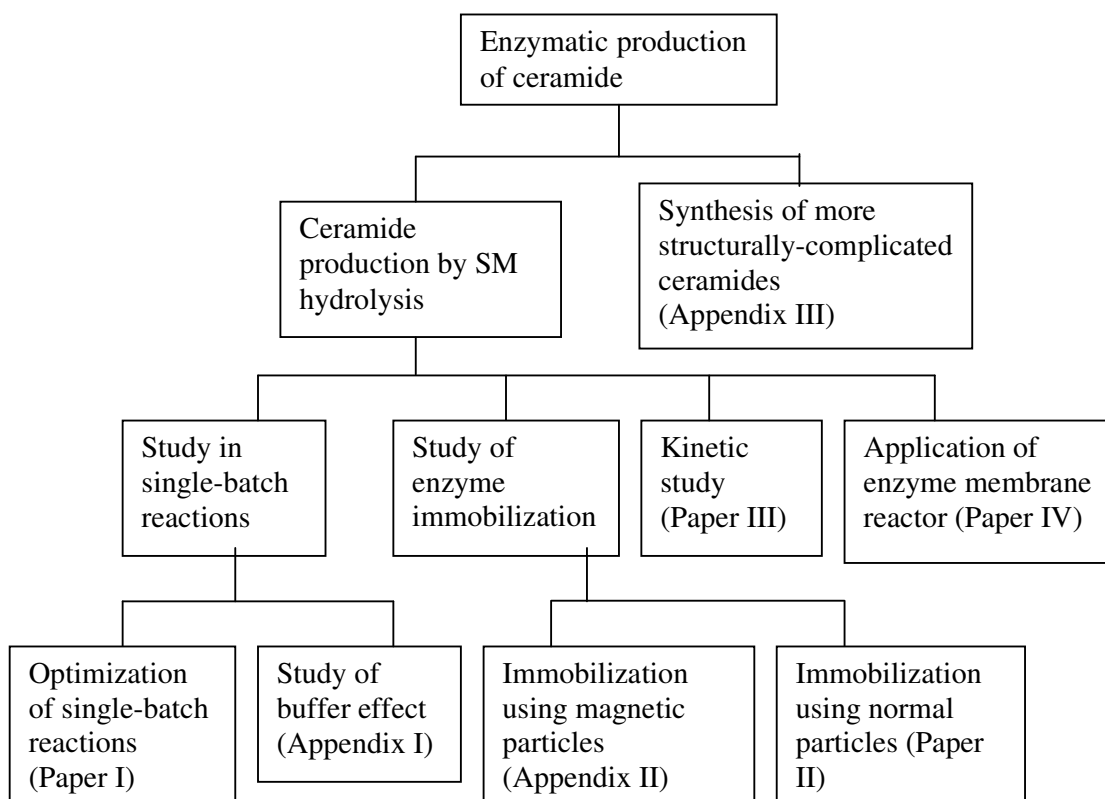


Figure 9: Overview of the experimental approach in the thesis. Related papers and appendices are included in the figure.

4.1 SM hydrolysis for ceramide production

As discussed previously, SM hydrolysis might be a feasible and profitable method for ceramide production. The enzymes used for catalyzing SM hydrolysis were SMase and PLC, which were commercial preparations. Although SMase is specific for the hydrolysis of SM, it is much more expensive than PLC. Therefore, it is our intention to screen and find a suitable PLC as a replacement. At the beginning of the study, commercial SM extracted from chicken egg yolk and bovine brain were used as substrates. These commercial products have a high purity (99 %), which ensures the repeatability and reliability of results from the feasibility investigation and the kinetic study. However, the price of commercial SM is so high that large scale production of ceramide through the hydrolysis of commercial SM would be very costly. To find a substitute, we purified SM from Lacprodan PL-75 (whey-fat phospholipid fraction from Arla Foods Ingredients a.m.b.a., Viby J. Denmark) by mild alkaline hydrolysis. The purity of SM in final product was 78 %, and the recover rate was 80 %. Consequently, we can get a large amount of SM through purification, making the large scale production of ceramide possible. The purified SM was used as substrate in the enzyme membrane reactor investigation (Paper IV).

The reaction volume varied from 0.2 ml to 79 ml, according to different features of individual studies. For example, since the kinetic study dealt mainly with possible reaction mechanisms, the reaction time had to be precise, and the number of tests was large. With adequate shaking of the reaction mixture, the reaction having a smaller volume (0.2 ml) would be convenient in practice, and was a better choice in the kinetic study. Results showed that the catalytic activity of the enzyme was almost the same when the reaction volume was decreased from 2 ml to 0.2 ml. In contrast, the enzyme membrane reactor was able to combine hydrolysis with product purification, and to improve the stability of the enzyme. Due to the potential for improving large scale production of ceramide, the study on the enzyme membrane reactor was carried out at a larger reaction volume (79 ml). In this way, the advantages of using the enzyme membrane reactor were more convincing to the readers.

4.1.1 SM hydrolysis in single-batch reactions (Paper I and Appendix I)

The reactor used was a flat bottom vial with an aluminum crimp cap for sealing (Fig. 10). The reaction volume was 2 ml. Firstly, SM hydrolysis in water-saturated toluene and in a two-phase (water: toluene) system were conducted and compared, with three types of PLC as catalysts. In the two-phase system, one SMase and four PLC were screened for SM hydrolysis, and one PLC was selected for further study. The effects of temperature, pH and ethanol on SM hydrolysis were evaluated. A fractional factorial resolution III design based on the principles of response surface methodology (RSM) was used to evaluate the effects from three compounds: $(\text{NH}_4)_2\text{SO}_4$, NaCl and CaCl_2 . The only response was the ceramide concentration (mg ml^{-1}) after 40 min reaction time. Seven experimental settings were generated by the applied software Modde 6.0 (Umetrics, Umeå, Sweden).



Figure 10: The used enzyme reactor.

Different types of organic solvent were compared in SM hydrolysis under the reaction conditions suggested from the previous study. To determine the optimal conditions for ceramide production, a five-factor central composite design circumscribed with star distance 2 was used according to the principles of RSM. The number of experimental settings generated by the applied software Modde 6.0 was 29. The factors and their ranges applied were: enzyme amount (1-9 U per reaction mixture), water concentration (1.5-7.5 %), ethanol concentration (0.3-2.7 % final concentration in mixture), reaction time (30-90 min) and the ratio of hexane in ethyl acetate (30-70 %). Ranges for the factors were set based on the preliminary studies. The only response was the ceramide concentration. Experimental data were analyzed by a response

surface regression procedure to fit second order equation. The details of RSM and data analysis are presented in Section 4.3. The hydrolysis performances using two types of buffers were compared, and the influence of the buffer concentration was investigated in SM hydrolysis. Further information on the experiments can be obtained in Paper I and Appendix I.

4.1.2 Study of enzyme immobilization (Paper II and Appendix II)

The immobilization of *Clostridium perfringens* PLC was studied and the catalytic properties of the immobilized enzyme were investigated in SM hydrolysis to produce ceramide. The immobilization efficiencies and the activities of the immobilized enzyme were compared using nine different carriers. The effects of ethanol and the initial enzyme concentration on the immobilization of the enzyme were also evaluated. The hydrolysis reaction catalyzed by the immobilized PLC was optimized based on the principles of RSM. A three-factor central composite design circumscribed with star distance 1.682 was utilized according to the principle of RSM. The number of experimental settings generated by applied software Modde 6.0 was 17. The factors and their ranges applied were: temperature (21.9-52.1 °C), added water volume (35.9-204.1 µl buffer solution containing 25 % ethanol) and pH (7.53-8.87). The only response was the initial reaction rate ($\mu\text{g min}^{-1}$). Experimental data were analyzed by a response surface regression procedure in order to fit in the RSM model, whose details are presented in Section 4.3. Temperature and pH were also evaluated individually, with other parameters fixed at their optimal values. The operational and storage stabilities of the immobilized enzyme were examined under optimal conditions. The feasibility of applying magnetic particles for immobilization of the enzyme was also investigated for SM hydrolysis. Two types of magnetic particles were used in the immobilization process, and the results of their immobilization were compared. Further information on the experiments can be obtained in Paper II and Appendix II.

4.1.3 Kinetic study (Paper III)

To elucidate possible reaction mechanisms and to further improve hydrolysis, the kinetic performance of SM hydrolysis in the optimal two-phase (water: organic solvent) reaction system was investigated. The reactor used was a small glass tube (inner diameter, 0.9 cm; height, 7.4 cm, Fig. 11). Thermal stabilities of the enzyme powder, the enzyme in solution, and the enzyme immobilized on particle carriers were studied, whereby the effects of both the heating temperature and heating period on the catalytic activities were evaluated. The effects from the concentrations of the enzyme and the substrate were further examined in the hydrolysis. The kinetic parameters in Michaelis-Menten kinetics were calculated. The influence of ceramide addition on SM hydrolysis was investigated. More information on the experiments can be obtained in Paper III.

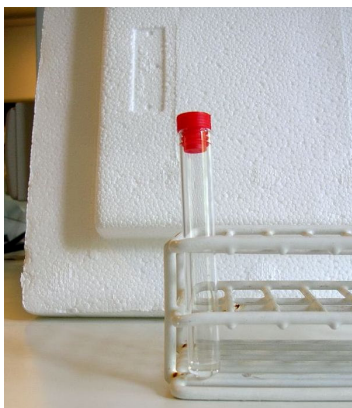


Figure 11: The reactor used in the kinetic study.

4.1.4 Application of enzyme membrane reactor (Paper IV)

An enzyme membrane reactor (Fig. 12) for the production of ceramide through SM hydrolysis with PLC from *C. perfringens* was studied. In the membrane reactor, the aqueous phase and the organic phase were separated by a membrane containing the immobilized enzyme, while the organic phase was continuously circulated. The enzyme was immobilized in ten different membranes to test the immobilization efficiency and the catalytic activity towards SM hydrolysis. Three immobilization

methods, filtration (adsorption/entrapment), covalent binding and cross-linking, were compared. The effects from the operation number of filtration and washing were examined to optimize immobilization process. In order to characterize SM hydrolysis in the enzyme membrane reactor, the effects of organic phase flow rate and enzyme load were evaluated, and operational stability was also examined. Finally, the soluble enzyme, enzyme immobilized on particle carriers, and enzyme immobilized onto membranes were compared to illustrate the membrane reactor's distinct features compared to more conventional approaches. More information on the experiments can be obtained in Paper IV.

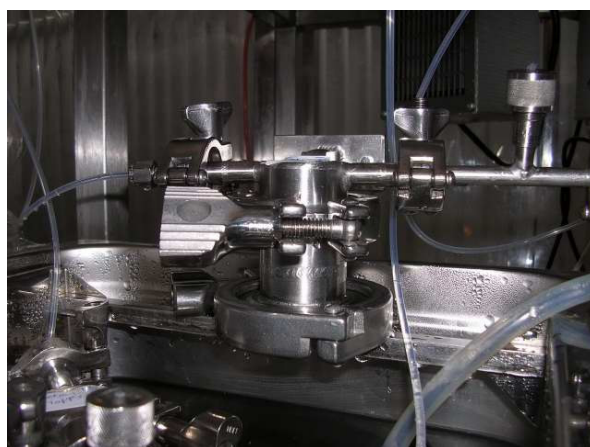


Figure 12: The enzyme membrane reactor setup.

4.2 Enzymatic synthesis of more structurally-complicated ceramides (Appendix III)

Due to their complex structure, some natural ceramides, such as ceramide 1 and ceramide 4, have yet to be synthesized. Their important functions in skin structure and in the maintenance of skin health have been recognized in many studies (Choi and Maibach, 2005; Bouwstra and Poncet, 2006; Coderch *et al.*, 2003). With the aim to produce these ceramides, several schemes had been proposed for the synthesis of ceramide 1. All the schemes include a key step, the enzymatic acylation of sphingosine, which was investigated further through a series of experiments. Sphingosine and radioisotope-labelled palmitic acid were used in the acylation

reaction catalyzed by human neutral ceramidase (EC 3.5.1.23). The effects of the reaction time and the sphingosine concentration on the acylation were evaluated in a micelle-in-water system. More information about the background and the experimental approach can be found in Appendix III.

4.3 Analysis of experimental data

When designing experiments for the evaluation of various reaction parameters, two methods were used: The first was the conventional approach, whereby one parameter was varied at a time while keeping all other parameters constant. This method was used most during experimental investigations. Data analysis using this approach was simple, and thereby further description in the thesis is not necessary. The second method involved applying the principles of RSM to set up a model, whereby the effects of several parameters and their interactions were evaluated at the same time. RSM and the data analysis process are introduced in detail.

RSM is an effective statistical technique for optimization of complicated systems, which enables the evaluation of the effects from multiple parameters, alone or in combination, on response variables. If the design and the selected ranges of the factors are appropriate, a surface that possibly contains the optimal points can be obtained for each response. The main advantage of RSM is the reduced number of experiments needed to provide sufficient information for statistically acceptable results (Xu *et al.*, 1998). With the assistance of several commercial programs, RSM are widely used by many researchers in the optimization of different systems. However, it should be mentioned that models can only be used in the defined ranges of each factor for optimization or prediction, and extrapolation is impossible. Models evaluate effects on response variables through multiple parameters, and evaluation of the effects from every factor will influence each other, even though in a good model, the predicted value is almost the same as the observed value.

The data were analyzed by means of RSM with software MODDE 6.0 (Umetrics, Umeå, Sweden). Second-order coefficients were generated by regression analysis with backward elimination. Response was first fitted to the factors by multiple regressions.

The quality of fit of the model was evaluated by the coefficients of determination (R^2) and the analysis of variances (ANOVA). Models with acceptable qualities should have $R^2 > 0.8$. The insignificant coefficients were eliminated in a stepwise fashion after examining the coefficients, and the model was finally refined. The quadratic response surface model was fitted to the following equation:

$$Y = \beta_0 + \sum_{i=1}^n \beta_i x_i + \sum_{i=1}^n \beta_{ii} x_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^n \beta_{ij} x_i x_j + \varepsilon \quad (1)$$

where Y is the response variable, x_i the i th independent variable, β_0 the intercept, β_i the first-order model coefficient, β_{ii} the quadratic coefficient for the variable i , β_{ij} the model coefficient for the interaction between factor i and j , ε the combination of the experimental error for the factors, and n is the number of the factors.

4.4 Sample analysis

Different analytical methods were applied during the present study. The primary analytical method employed, HPTLC (high performance thin-layer chromatography), is discussed here, while the information for other analytical methods may be found in the papers or appendices. Due to its intrinsic properties, HPTLC has become more popular for the analysis of lipid mass. In HPTLC, distinct from TLC (thin-layer chromatography), plates are made of fine particles, which have a narrow size distribution and excellent resolving power. HPTLC has allowed for the use of less volume of sample and development solvent, and more rapid development time (Sherma and Fried, 1991). Together with the use of in-situ densitometry for lipid quantification, HPTLC is a convenient and inexpensive method for lipid analysis.

The HPTLC used employed normal phase chromatography, which separated lipids according to their different polarities. Silica gel was the stationary phase and the development solvent was the mobile phase. Following the development of the sample, a charring reagent, cupric sulfate-phosphoric acid, was used to detect the lipids on the HPTLC plate. Lipids were visualized by heating the plate at 160 °C for 6 min. Samples were quantified by an in-situ densitometer. Standard samples were applied

and run on the same plate. The lipid mass was calculated from analyzing the peak area using the standard curve. If the peak area was in the standard curve, the relevant amount of the lipid in the sample could be calculated. Fig. 13 is a flow chart of the analysis process using HPTLC. A more detailed analytical procedure can be found in the papers (Paper I, II, III, IV).

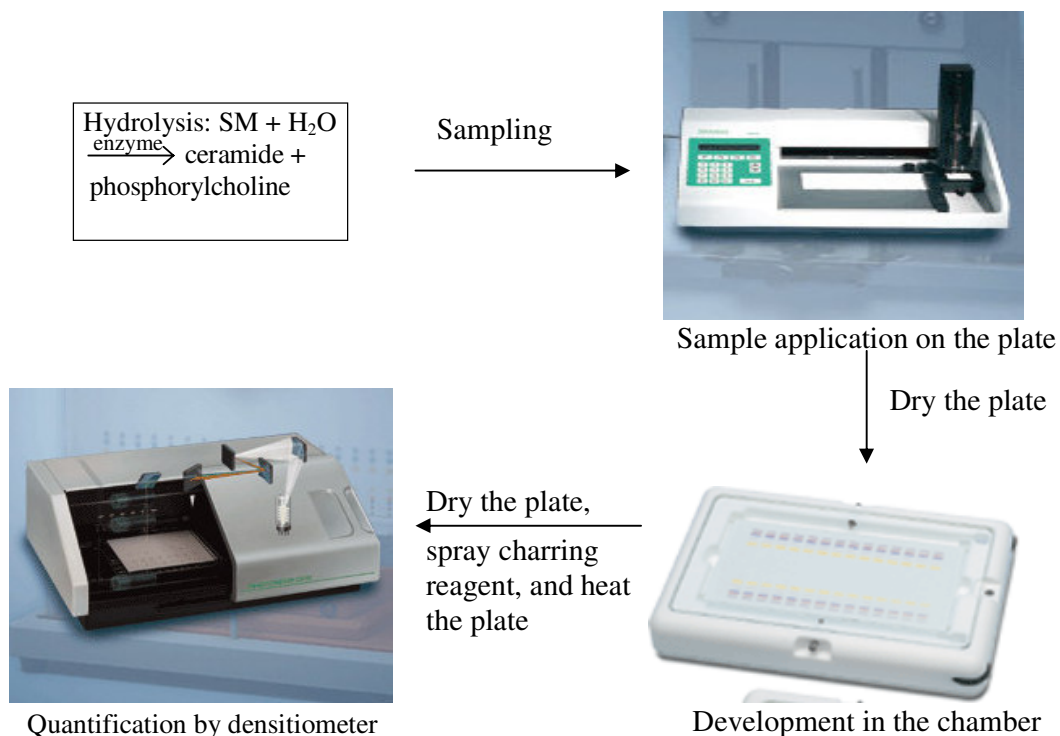


Figure 13: The flow chart of the analysis process using HPTLC.

From the HPTLC plate (Fig. 14), ceramide was visualized as double bands close together, in samples and standards. This pattern may have formed as a result of the acyl chain composition. When natural SM and synthesized SM were developed by other solvent systems, the similar double-band pattern emerges and is clearly dependent on the acyl chain composition (Ramstedt *et al.*, 1999; Ramstedt and Slotte, 1999).

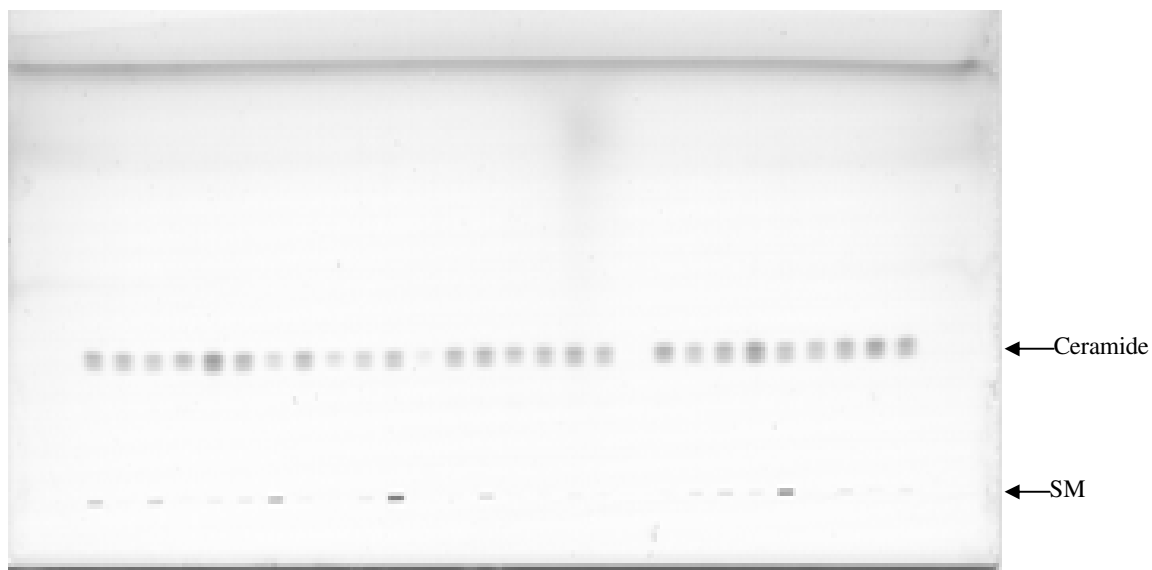


Figure 14: The HPTLC plate for scanning. The development solvent system was heptane: isopropanol: acetic acid (85: 15: 1). The lipids in the plate have been visualized by spraying with the charring reagent and heating. The bands are labeled by the representing lipids in the figure.

5 Summary of experimental work

This chapter presented the summary of experimental work in the enzymatic production of ceramide, and some brief discussions on the results. For more detailed information, please refer to the individual papers and appendices.

5.1 SM hydrolysis in single-batch reactions (Paper I and Appendix I)

Systematic investigation and optimization of SM hydrolysis for the production of ceramide was conducted in single-batch reactions. The reaction was more efficient in a two-phase (water: organic solvent) system than in a one-phase (water-saturated organic solvent) system. The existence of a water phase enhanced SM hydrolysis, indicating that water not only played a role as a nucleophile, but also contributed to the active state of the enzyme. PLC from *B. cereus* had the lowest activity towards SM hydrolysis. Among the PLC screened, PLC from *C. perfringens* had the highest catalytic activity, and hereby was the selected enzyme used for later studies. SM hydrolysis had a small temperature dependence, even though the highest SM conversion occurred at 37 °C. The reaction had two pH peaks at pH 8.6 and pH 7.4, with the highest ceramide production at pH 8.6. The existence of two pH peaks in the hydrolysis could be the result of: 1. Isomers or two enzymes that have similar substrate specificity probably exist in the PLC commercial product; 2. PLC from *C. perfringens* have two domains responsible for the binding step and the subsequent catalytic step, respectively (Clark *et al.*, 2003). If these two steps possess different favorable pHs due to the relative functional groups present in the different domains, then the apparent pH plot would display the combined pH influences.

The addition of (NH₄)₂SO₄, NaCl and CaCl₂ in the reaction mixture had no significant effect on the hydrolysis. Since the enzyme product from the commercial source wasn't pure protein, these compounds might have already been in the enzyme product. This could be the reason for discrepancies with the literature (Krug and Kent, 1984). Addition of ethanol to the system enhanced the rate of ceramide formation.

Ethanol addition might increase the solubility of SM and ceramide, thereby enhancing the rate of diffusion to and from the active site. Ethanol could also induce exposure of histidine groups in the enzyme, as found in a study using *B. cereus* PLC (Little, 1977b), where histidine groups in the active site played a crucial role in catalytic properties. The use of ethyl acetate: hexane (50:50, v/v) as the reaction medium improved SM hydrolysis. This could be caused by an increase in the solubility of ceramide or water in the organic solvent system.

Based on the results from the above studies, the hydrolysis was optimized using RSM with five factors (enzyme load, water content, ethanol content, reaction time and the ratio of hexane in ethyl acetate, detailed information on the range of factors and RSM was presented in Sections 4.1.1 and 4.3). In the fitted quadratic model, water content and enzyme content had the most significant influence on the hydrolysis reaction, followed by reaction time. Through iterative calculations with the target of maximum ceramide concentration, RSM results suggested 75 min reaction time, 3 U mL⁻¹ enzyme content, 6 % water content, 1.8 % ethanol content and 46 % hexane in ethyl acetate as optimal conditions for ceramide production. Employing these conditions, the predicted ceramide concentration was 0.68 mg mL⁻¹, where 88 % of SM was converted to ceramide. SM hydrolysis using Tris-HCl buffer had a higher reaction rate than using a mixture buffer having a broad range from pH 2 to pH 12. Some compounds, such as phosphate and citrate, in the mixture buffer may have had some inhibitory effects on the enzyme. The much higher ionic strength of the mixture buffer (compared with Tris-HCl buffer) may have also lead to a decreased hydrolysis rate. Increasing the concentration of Tris-HCl buffer in the enzyme solution had a negative effect on SM hydrolysis.

Based on systematic research in single-batch reactions, SM hydrolysis was found to be a feasible approach to the potential production of ceramide. After system evaluation and optimization of several important factors, the performance of the hydrolysis was dramatically improved. Consequently, the results obtained from this study provided valuable information for further development and applications.

5.2 Enzyme immobilization for SM hydrolysis (Paper II and Appendix II)

Immobilization can improve enzyme reusability, and offers both better process possibilities and efficiency. The immobilization of *C. perfringens* PLC was studied and the catalytic properties of the immobilized enzyme were investigated for SM hydrolysis to produce ceramide. By screening nine different commonly-used carriers, the highest immobilization efficiency (96 %) was found for Amberlite® XAD7. Enzyme immobilized on the carrier, Lewatit VP OC 1600 (Lew), had the highest catalytic activity towards SM hydrolysis, even though only 25 % of the enzyme was immobilized on this carrier. Lew is a divinyl benzene crosslinked polymer (matrix: methacrylate). The high activity of the enzyme immobilized on Lew was probably due to Lew's hydrophobic properties. The key amino acids in the active site of *C. perfringens* PLC are hydrophilic (Naylor *et al.*, 1998). If the carrier was also hydrophilic, a large risk would exist for the carrier to interact with the active site of the enzyme, effectively blocking the active site. In addition, since both the substrate and the product are hydrophobic molecules, the hydrophobic surroundings of the enzyme in Lew particles could enhance substrate and product transfer to and from the active site.

Pre-wetting Lew with ethanol increased the amount of the enzyme immobilized on the carrier, but both specific and apparent activities of the immobilized enzyme were decreased. The carrier swelled by wetting with ethanol, resulting in increased pore size of the matrix. Consequently, the accessible surface area (the surface area where the enzyme can be approached during the immobilization) of the carrier was increased. Usually, the larger the accessible surface area, the greater the contact between the enzyme and the carrier, and the higher the extent of inactivation that can be expected (Barros *et al.*, 1998). Increasing the initial enzyme concentration resulted in more enzyme adsorption on the carrier and a higher specific activity for the enzyme. The reasons for the increase of the enzyme specific activity were proposed as follows: 1. Certain “dead pores” exist in the carrier, and the enzyme can be inactivated through contact with these dead pores. When the amount of the enzyme immobilized on Lew was increased, these dead pores were saturated by the enzyme.

After the saturation point, the new adsorbed enzyme exhibited the intrinsic activity. 2. Since the amount of the carrier was unchanged, the accessible surface area of the carrier was fixed. The increase in the amount of the enzyme in Lew decreased the accessible surface area per enzyme molecule, leading to less contact between individual enzyme molecule and Lew. As a result, the extent of inactivation caused by this contact was reduced.

The hydrolysis reaction catalyzed by the immobilized PLC was optimized with the assistance of RSM for three factors (temperature, water content and pH, detailed information presented in Sections 4.1.2 and 4.3). In the fitted quadratic model, temperature and water content had significant influence on SM hydrolysis, and the reaction had little dependence on pH. The optimal temperature was around 46 °C, and the optimal water content was 3.5 %. From analysis of the interaction between temperature and water content, it was found that high water content made the enzyme more vulnerable to thermal deactivation. When the effects of temperature and pH were further evaluated individually, the results obtained were in accordance with the model. The soluble enzyme was difficult to reuse under present conditions. During the recycling, the immobilized enzyme retained a greater portion of its original activity at 37 °C compared with the same reaction at 46 °C, confirming that thermal inactivation played an important role in the reuse of the enzyme. Since the optimal temperature was around 46 °C for hydrolysis catalyzed by the immobilized enzyme, a compromise has to be made between the reaction rate and the reusability of the enzyme. At a reaction temperature of 37 °C, the immobilized enzyme retained around 70 % of its initial activity after 7 cycles. When both the dissolved PLC and the immobilized PLC were stored at room temperature, the activities of the enzymes were irregular after different storage periods. The immobilized PLC followed first order deactivation with the storage at 40 °C. The characteristic half time for thermal inactivation was 4.1 days, and K_D was 0.17 per day. When magnetic particles, M-PVA N12 and BLM, were used in the immobilization, more than 60 % of the enzyme was immobilized onto the particles. Although there is a great advantage for the application of magnetic particles for immobilization, the specific activity of the immobilized enzyme was too low compared with the soluble enzyme.

As discussed above, the enzyme immobilized on Lew had the highest catalytic activity towards SM hydrolysis. The geometric characteristics of the carrier were a crucial factor that influenced the observed catalytic activity of the immobilized enzyme. Based on the results from the optimization study and stability testing of the immobilized enzyme, the performance of SM hydrolysis can be improved through enzyme immobilization. Based on this, the application of the immobilized PLC to produce ceramide through SM hydrolysis seems attractive.

5.3 Kinetic study for SM hydrolysis (Paper III)

Based on the optimal reaction conditions obtained from previous work, the kinetic study of SM hydrolysis catalyzed by *C. perfringens* PLC was conducted for the improvement of ceramide production. In order to test thermal stability, the enzyme powder, enzyme dissolved in solution, and enzyme immobilized onto particle carrier (Lew) were heated before the reaction. The enzyme dissolved in solution was most vulnerable to increases in temperature. At a temperature of 65 °C, the catalytic activities of the enzymes in all three forms decreased with the prolongation of the heating time. The enzyme dissolved in solution was denatured very quickly by heating. The enzyme powder was less affected by the heating time than the immobilized enzyme. The thermal inactivation of the enzymes in all three forms did not follow first order reaction kinetics at a temperature of 65 °C. Investigation of different heating periods showed thermal inactivation of the immobilized PLC following first order reaction kinetics between 0 and 40 min. Hence, the heating period was also found to affect thermal inactivation. For the soluble enzyme, the initial reaction rate was proportional to the enzyme concentration in the range investigated. Therefore, the current reaction system could be a new method for the catalytic activity assay of PLC.

Following evaluation of the effect of substrate concentration, the reactions for both the soluble enzyme and the immobilized enzyme followed Michaelis-Menten kinetics. K_m s for the soluble enzyme and the immobilized enzyme were 1.07 ± 0.32 and 1.26 ± 0.19 mM, respectively. Immobilization on particle carriers markedly decreased the value of V_{max} without much change in K_m , in a manner similar to non-competitive inhibition. The carrier possibly contacted the enzyme molecule not mainly through the

active site of the enzyme. The decrease of V_{\max} might be the result of reduced mobility of the immobilized enzyme. However, the reduction of V_{\max} for the immobilized enzyme without much change in K_m may have also been due to other factors. After immobilization, some enzymes might retain the same values for K_m and V_{\max} as the soluble enzyme, while other enzymes might lose most of the catalytic capability. Therefore, the apparent K_m only showed the K_m of the former enzymes, where the value would be similar to the soluble enzyme. Since the total catalytic activity was reduced by the immobilization, V_{\max} was significantly decreased.

When the product, ceramide, was added before the reaction, the initial reaction rate of SM hydrolysis increased. The effect of ceramide concentration on the hydrolysis was not linear, and a certain ceramide concentration was required to get a significant activation effect on the reaction. The phenomenon of product activation is very rare in enzymatic reactions. Since SM is an amphiphilic molecule, it is easy to congregate in the organic solvent. Ceramide could interact with SM through their hydroxyl groups which are able to form hydrogen bonds. SM solubility and rate of diffusion might be increased by the interaction with ceramide. Therefore, the addition of ceramide could assist SM molecule approach to the enzyme at the interface. Another possibility is that ceramide might stabilize the enzyme at the interface. PLC has a hydrophobic surface area near the active site (Cornell and Arnold, 1996), and ceramide is a hydrophobic compound. Through interacting with the hydrophobic surface area of PLC, ceramide might stimulate the aggregation of PLC molecules at the interface. Thus, more PLC could stay at the interface to catalyze the reaction. With increasing initial ceramide concentration, K_m decreased rapidly, and V_{\max} did not change significantly. This seems to be a reversal of the effect from competitive inhibition. The large change in K_m indicated that the addition of ceramide was the primary cause of the increased affinity of the enzyme-substrate complex.

5.4 Application of enzyme membrane reactor for SM hydrolysis (Paper IV)

An enzyme membrane reactor was set up for SM hydrolysis catalyzed by *C. perfringens* PLC. Ten flat sheet membranes were tested in membrane screening experiments, with the filtration as the immobilization method. The enzyme solution was filtered through a support layer to the active layer of the membrane during the immobilization process. Because the pore size of the support layer is much larger than the active layer, the enzyme was either entrapped or adsorbed onto the porous structure in the membrane instead of on the membrane surface. The amount of immobilized protein in all membranes showed little correlation with their molecular weight cut-off (MWCO) values. This could be a result of the diversity in protein molecular weights in the enzyme product from the commercial source. The special filtration direction opposite to normal filtration mode probably also contributed to the absence of MWCO value effects on the immobilization. The most hydrophilic membrane tested, RC 70PP (the active layer was made of regenerated cellulose acetate), had a low immobilization efficiency (9.4 %). The enzyme immobilized in membrane RC 70PP retained the highest specific activity. From the previous study (Paper I), SM hydrolysis was more efficient in a two-phase system where the enzyme at the liquid-liquid interface had a higher catalytic activity. Due to the hydrophilicity, the active layer of RC 70PP was easily immersed in water. Consequently, the interface between the water phase and the organic solvent phase lay deep inside the membrane. Most enzymes were in contact with the interface, making catalysis more efficient. In other hydrophobic membranes, the interface remained near (or on) the membrane surface, towards the aqueous phase. Most enzymes in the membrane were surrounded only by the organic solvent, as in the one-phase system where the activity of the enzyme was much lower than in the two-phase system.

To improve the immobilization efficiency, other immobilization methods, covalent binding (for RC 70PP) and cross-linking (for membranes E1004/TX and GR 90PP) were investigated. The application of these methods increased the immobilization efficiency at least 5 times. However, both of the specific activities of the enzyme and the SM conversion ratio after 54 h reaction time decreased dramatically in cross-

linking and covalent binding methods. The amino groups of the enzymes were covalently attached to the membrane (in covalent binding) or to other amino groups of the enzymes with glutaraldehyde (in cross-linking). These could alter the protein tertiary structure, causing a loss in the activity of the enzyme (Hilal *et al.*, 2006). Moreover, these two methods provided higher enzyme loading and more rigid attachment of the enzyme in the membrane than adsorption or entrapment through filtration. This might also increase the mass transfer limitation. In the optimization of the filtration process, the amount of the immobilized protein significantly increased in the first two batches of filtration, and reached a plateau in the following batches. The immobilization efficiency was only slightly affected by the number of washings, indicating that the immobilization was relatively stable.

For SM hydrolysis in the enzyme membrane reactor, the optimal flow rate of the organic phase was 5 ml/min. The increase of the flow rate enhanced the diffusive transport of SM from the organic phase bulk to the reaction microenvironment and ceramide in a reverse direction. However, a high flow rate could lead to a pressure rise in the organic phase, which in turn pushes the interface of the two liquid phases in the membrane towards the water phase. The contact between the interface and the enzyme in the membrane was limited, thereby inhibiting SM hydrolysis. Immobilization of a high initial enzyme load led to a decrease in immobilization efficiency. The membrane was probably saturated with the loaded protein. Both the initial reaction rate and the specific activity of the enzyme increased with increased enzyme loading, and decreased slightly when the enzyme load surpassed 50 μg on a 9.6 cm^2 membrane area. During filtration, some enzyme was inevitably located away from the interface of the two liquid phases and exhibited little activity. With low enzyme loading, the fraction of the inactive enzyme was higher, and thus the specific activity calculated based on the total immobilized enzyme was low. When a high amount of the enzyme was immobilized, the porous structure located away from the interface might have already been saturated by the enzyme, and the ratio of the inactive enzyme to the total loaded enzyme possibly became much smaller. Thus, the specific activity derived from the immobilized enzyme increased.

The enzyme immobilized in the membrane retained 16 % of its original activity after 5 cycles. In Paper II, PLC immobilized on the particle carrier retains 70 % of its initial

activity after 7 cycles. However, in that study, the reaction time (20 min) for each batch and the reaction volume (2 ml) were largely different from the current study (24 h and 79 ml, respectively). Therefore, the results from these two studies are not comparable. Loss of activity during reuse might be caused by enzyme denaturation or twisting of the membrane pore structure during the long operation period. To illustrate the membrane reactor's distinct features, SM hydrolysis using the liquid enzyme, enzyme immobilized on particle carriers, and the enzyme immobilized in the membrane were compared. The membrane reactor developed showed improved enzyme reusability, a fast immobilization process, straightforward up-scaling and the ability to combine hydrolysis with product separation. Therefore, SM hydrolysis in the enzyme membrane reactor was a feasible approach for ceramide production. Based on the fact that the enzyme activity was higher in the two-phase system than in the one-phase system, the relationship between the enzyme localization and the interface of the two liquid phases had a significant effect on the catalytic activity, and played a crucial role during the evaluation of reaction parameters, including membrane type, flow rate of the organic phase and the enzyme load, etc.

5.5 Assessment of various approaches for SM hydrolysis

Up to now, SM hydrolysis for ceramide production had been studied in a single-batch reaction, in a reaction with the enzyme immobilized onto particle carriers, and a reaction with the enzyme immobilized onto a membrane. It would be necessary to distinguish their intrinsic characteristics through an assessment of these approaches. Part of this work has already been done in the individual papers. Nevertheless, due to its significance for future applications, a summary of the assessment and comments on the various approaches in more detail are presented here.

The single-batch reaction using the liquid enzyme was the simplest and most convenient method. Comprehensive investigations were conducted in the single-batch reaction, and the results obtained provided a basis for the other approaches and further development. Using this method, the enzyme exhibited a much higher specific activity than with the other approaches. However, it is difficult to reuse the liquid enzyme in the reaction. Since the price of PLC is high, this would hinder further development

and applications at an industrial level. Thus, it is necessary to improve the reusability of the enzyme, which would significantly reduce the cost during large-scale production of ceramide.

In spite of the low specific activity, the reusability of the enzyme was greatly improved through immobilization techniques. In the reaction with the enzyme immobilized onto particle carriers, PLC retained around 70 % of its initial activity after seven cycles. Other benefits of this approach were that the reaction catalyzed by the immobilized enzyme had little dependence on pH and had a higher temperature optimum than the reaction catalyzed by the liquid enzyme. Due to the pH tolerance, the reaction could be conducted within a broad pH range, subject to the specific preference. In addition, utilization of the PLC immobilized onto particle carriers was flexible, i.e. the immobilized enzyme could be used in both batch and continuous stirred-tank reactors, at various scales.

The enzyme immobilized onto the membrane had a similar specific activity to the enzyme immobilized onto particle carriers. Enzyme immobilization on the membrane through filtration was faster than immobilization onto particle carriers, indicating that membrane immobilization was a more convenient method. Besides, up-scaling of the enzyme membrane reactor was straightforward and allowed for continuous ceramide production. Since phosphorylcholine is soluble in the aqueous phase and ceramide can be dissolved in the organic phase, they would be separated at the interface of the two liquid phases by phase extraction during the reaction. This combination of SM hydrolysis and product separation would also be an advantage during application of the enzyme membrane reactor. Therefore, the employment of the enzyme membrane reactor could have been a good opportunity for improvement of the reaction performance. However, the set up of the enzyme membrane reactor was more complicated than those used for the other reactions. Parameters, such as the available membrane area and the shape of the reactor, were difficult to change after assembly. As a result, some efforts are still required in the design of the enzyme membrane reactor.

5.6 Enzymatic synthesis of more structurally-complicated ceramides (Appendix III)

In order to evaluate the possibility to produce more structurally-complicated ceramides, such as ceramide 1 and ceramide 4 (Fig. 15), four schemes had been proposed for the synthesis of ceramide 1. Due to the complicated structure, the synthesis of ceramide 1 is very challenging and involves a lot of work, as depicted by the schemes. The current study is only in “proof of concept” stage. In order to produce ceramide 1, the proposed schemes should be investigated and compared experimentally:.

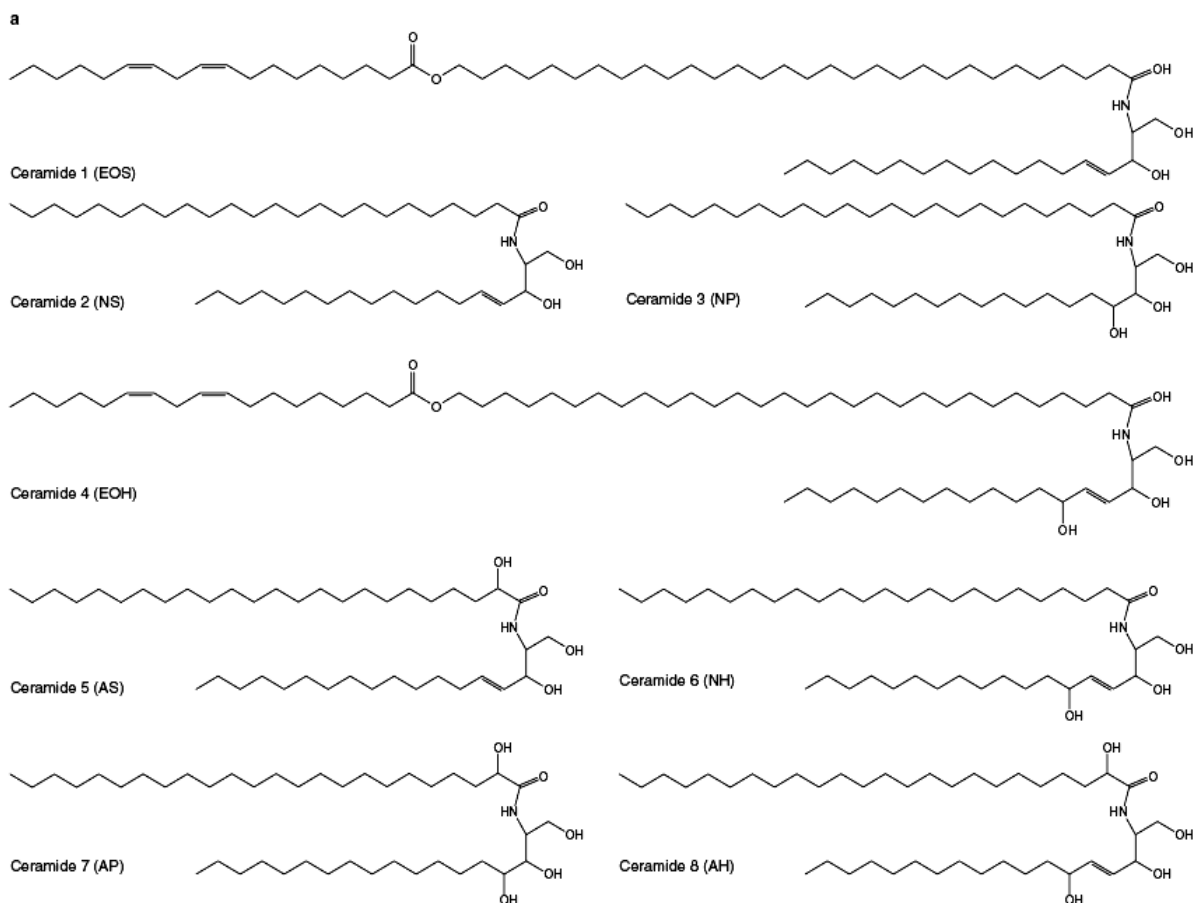


Figure 15: The main ceramides present in the human stratum corneum (Corderch *et al.*, 2003).

All the schemes include a key step, the enzymatic acylation of sphingosine to produce ceramide. This step was investigated further through designed experiments. Sphingosine and radioisotope-labelled palmitic acid were used in the acylation reaction catalyzed by human neutral ceramidase. A 50 % yield was achieved after a 24 h reaction time. The ceramide production increased linearly with the rise of the sphingosine concentration. As such, we could conclude that the enzymatic acylation of sphingosine was a viable method for the production of a simple-structured ceramide on a bench scale. Consequently, current results could form the basis for further studies in the synthesis of ceramide 1.

6 Conclusions and future perspectives

The studies in the present thesis provide a better understanding of some important aspects concerning the enzymatic production of ceramide. Enzymatic hydrolysis of SM was proven to be a feasible approach for potential production of ceramide. Through systematic research from the single-batch reaction to the continuous production, and from 0.2 ml reaction volume to 79 ml reaction volume, efficient and scalable production methods were developed for industrial applications. The main achievements of the project were summarized in the following paragraphs.

Through systematic evaluation and optimization of several factors in single-batch reactions, the performance of SM hydrolysis was dramatically improved. The reaction was more efficient in a two-phase (water: organic solvent) system than in a one-phase (water-saturated organic solvent) system. Among the enzymes screened, PLC from *C. perfringens* was found to have the most advantageous properties. The reaction had a slight temperature dependence and two pH peaks. Addition of ethanol to the system enhanced the rate of SM hydrolysis, and a mixture of ethyl acetate and hexane (50:50, v/v) was found to be the best organic solvent tested. When the hydrolysis was optimized based on the principles of RSM with five factors, water content and enzyme loading had the most significant influence on the hydrolysis reaction in the fitted quadratic model. SM hydrolysis using Tris-HCl buffer had a higher reaction rate than using a mixture buffer having a broad pH range from 2 to 12. Increasing the concentration of Tris-HCl buffer had a negative effect on SM hydrolysis.

In order to improve the reusability of the enzyme, the immobilization of PLC and the properties of the immobilized enzyme were studied. By screening nine commonly-used carriers, we found that the enzyme immobilized on Lew had the highest catalytic activity. Pre-wetting Lew with ethanol led to a higher immobilization efficiency, while the activity of the enzyme decreased. Increasing the initial enzymatic concentration resulted in more enzyme adsorption onto the carrier, and an increased specific activity. In the reaction catalyzed by the immobilized enzyme, the optimal temperature was around 46 °C, and the optimal water content was 3.5 %. The reaction had little dependence on pH. After 7 cycles, the immobilized enzyme retained around

70 % of its initial activity. The immobilized enzyme was deactivated irregularly when stored at room temperature, but followed first order deactivation when stored at 40 °C. When magnetic particles were used in the immobilization, more than 60 % of the enzyme was immobilized onto the particles, however, the specific activity of the immobilized enzyme was significantly decreased.

The kinetic performance of SM hydrolysis was investigated to elucidate possible reaction mechanisms and to further improve the hydrolysis. The enzyme dissolved in solution was less thermally stable than the enzyme powder or the enzyme immobilized on particle carriers. Thermal inactivation of PLC in all three forms did not follow first order reaction kinetics at a temperature of 65 °C. The reactions for both the soluble and immobilized PLC followed Michaelis-Menten kinetics. K_m s for the soluble and immobilized enzymes were 1.07 ± 0.32 and 1.26 ± 0.19 mM, respectively. The value of V_{max} was markedly decreased by immobilization onto particle carriers without much change in K_m , as if the immobilization functioned as non-competitive inhibition. Ceramide product activated the hydrolysis reaction. However, the kinetic study proved that its addition mainly caused an increase in the affinity of the enzyme-substrate complex.

In the study of the enzyme membrane reactor for SM hydrolysis, the enzyme was immobilized onto ten selected membranes, and the activity of the immobilized enzyme was tested in the two-phase reaction system. The enzyme immobilized on membrane RC 70PP had low immobilization efficiency, but retained the highest catalytic activity. When three immobilization methods, i.e. filtration (adsorption/entrapment), covalent binding, and cross-linking, were compared, the enzyme immobilized by filtration had the highest activity even with the low immobilization efficiency (9.4 %). The optimal flow rate of the organic phase was 5 ml/min. A high initial enzyme load in the immobilization led to decreased immobilization efficiency. Both the initial reaction rate and the specific activity of the enzyme increased with increasing enzyme load, but decreased slightly when the immobilized enzyme load was over 50 μg on 9.6 cm^2 membrane area. The immobilized enzyme retained 16 % of its original activity after 5 cycles. The membrane reactor system established for this reaction showed improved enzyme

reusability, a fast immobilization process, straightforward up-scaling and the ability to combine hydrolysis with product separation.

In the assessment of various approaches for SM hydrolysis, the benefits and drawbacks of each production method were discussed. Every approach had unique attributes, i.e. they each had their own advantages and disadvantages. The single-batch reaction using the liquid enzyme was the simplest and most convenient method for SM hydrolysis. The reactions with the enzymes immobilized onto particle carriers or onto membranes were more applicable to large scale production of ceramide.

Some more structurally-complicated ceramides can't be produced by SM hydrolysis. Several schemes had been proposed for their synthesis. Through designed experiments, the enzymatic acylation of sphingosine was proven to be a viable method for the production of simple-structured ceramide on a lab scale. A 50 % yield was achieved after a 24 hours reaction time. This reaction was a crucial step in all the proposed schemes, and hopefully will also be applicable in the synthesis of more structurally-complicated ceramides.

Even though the application of enzymes has many advantages, chemical synthesis is still the common method to produce ceramide. One of the reasons for this is the relatively high price of the enzyme. However, the interest in using enzyme processes and realization of their benefits have increased in the industrial sector. Consequently, this will encourage development and production of the enzyme in large quantities, and the price of the enzyme will eventually decrease. The enzymatic production of ceramide will become more competitive in the near future. Nevertheless, some problems other than the enzyme cost need attention before enzymatic production can be implemented on an industrial scale. In spite of the achievements in the present thesis, more work is still needed, as summarized below:

1. Ceramide purification from the reaction mixture is an important issue in large scale production. After the reaction, a certain amount of SM is left in the organic phase, and must be removed to increase the purity of the final product. Chromatographic methods have been applied in the separation between ceramide and SM for analytical purposes. Some work is required to validate

the chromatographic method for ceramide purification, or to find some alternative methods like membrane separation.

2. The current reaction system is on a bench scale. When the process is scaled up, additional difficulties for the enzymatic production of ceramide may occur, as is the case with most applications. Therefore, certain efforts to improve the feasibility and practicability are necessary in the up-scaling of the process.

7 Reference

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PAPER I

Long Zhang, Lars I. Hellgren and Xuebing Xu (2006) Enzymatic production of ceramide from sphingomyelin, *Journal of Biotechnology*, 123: 93–105

Enzymatic production of ceramide from sphingomyelin

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Abstract

Due to its major role in maintaining the water-retaining properties of the epidermis, ceramide is of great commercial potentials in cosmetic and pharmaceutical industries such as in hair and skin care products. Chemical synthesis of ceramide is a costly process, and developments of alternative cost-efficient production methods are of great interest. Present study was the first attempt to perform a systematic study on the production of ceramide through enzymatic hydrolysis of sphingomyelin. Sphingomyelin hydrolysis proved to be more efficient in two-phase (water:organic solvent) system than in one-phase (water-saturated organic solvent) system. Among the screened phospholipase C, the *Clostridium perfringens* enzyme had the highest sphingomyelin conversion rate, with very small temperature dependence. Addition of ethanol to the system markedly enhanced the rate of ceramide formation, and a mixture of ethylacetate:hexane (50:50) was the best organic solvent tested. Other factors such as $(\text{NH}_4)_2\text{SO}_4$, NaCl and CaCl_2 were also tested but excluded for further consideration. On the basis of the initial experiments, the reaction system was optimized using response surface methodology including five factors (enzyme amount, water amount, ethanol amount, reaction time and the hexane ratio of organic solvent). Water content and enzyme amount was shown to have the most significant influence on the hydrolysis reaction in the fitted quadratic model. The efficiency of sphingomyelin hydrolysis was dramatically improved through system evaluation and optimization, with the optimal conditions at 75 min reaction time, 3 U ml⁻¹ enzyme amount, 6% water amount, 1.8% ethanol amount and 46% hexane in ethylacetate.

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Keywords: Ceramide; Sphingomyelin; Phospholipase C; Optimization; Hydrolysis; Response surface methodology

1. Introduction

Ceramide (Fig. 1B), the *N*-acylated derivative of sphingosine, is the key intermediate in the biosyn-

thesis of all complex sphingolipids. Ceramide and its derivatives have raised a large interest as active components in both the pharmaceutical and cosmetic industries. Ceramide plays a major role in the water-retaining properties of the epidermis (Elimelech, 2002; Kenkyusho, 1995; Smeets et al., 1997), hence, it has been broadly used as moisture-retaining ingredients for human skin care products in the cosmetic industry. It is claimed that these products dramatically increase

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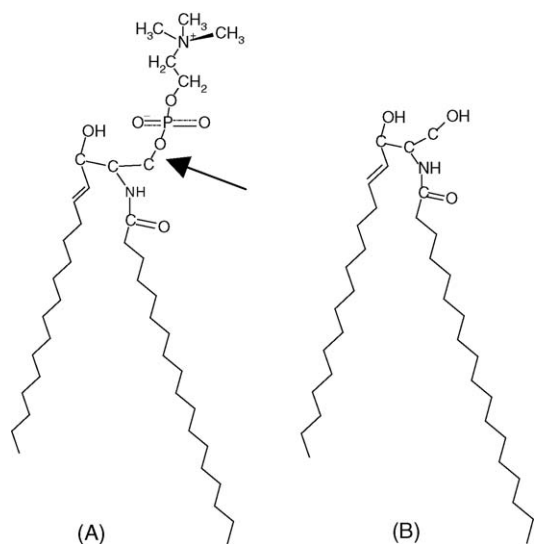


Fig. 1. The structures of sphingomyelin (SM) (A) and ceramide (B). The arrow indicates the cleave bond in SM for producing ceramide.

skin's hydration level, repair the cutaneous barrier, prevent vital moisture loss, and contribute to reducing dry flaky skin and aged appearance. Furthermore, a lotion containing 0.1% ceramide in a hydrophilic ointment has excellent curing effects on patients of atopic dermatitis (Susumu et al., 1999), implying the potential of ceramide as an active component in dermatological therapy.

Synthetic ceramide is normally obtained by acylation of the amine group of a sphingosine, a sphinganine or their derivative (Cho et al., 1995; Didier et al., 1997; Smeets et al., 1997). In several reported methods, preparing these materials relies on the use of serine, carbohydrates, L-glyceric or D-tartaric acids, etc. as a chiral building block (Liotta and Merrill, 1992). However, these synthetic approaches for the preparation of isomerically pure sphingosine and ceramide are tedious and time consuming for industrial applications (Elimelech, 2002). Therefore, it is desirable to find an alternative cost-efficient and high-yield method for obtaining this valuable product.

Sphingomyelin (SM) is a ubiquitous component of animal cell membranes and it is one of the major phospholipids in bovine milk. In SM, the ceramide part of the molecule is bound through a phosphodiester bridge to a choline moiety (Fig. 1A). So far, only few studies have focused on ceramide production using SM

hydrolysis (Kenkyusho, 1995; Susumu et al., 1999), and no systematic study has been published. Therefore, the aim of present work was to systematically investigate and optimize the enzymatic production of ceramide from SM.

The modification site for producing ceramide from SM is a bond between the primary hydroxyl group of ceramide and choline phosphate ester (Fig. 1). Since this bond cannot be broken specifically using chemical hydrolysis (Susumu et al., 1999), an enzymatic method must be applied. Possible enzymes for catalyzing the hydrolysis reaction are sphingomyelinase (SMase, EC 3.1.4.12), and phospholipase C (PLC, EC 3.1.4.3). SMase is a specific enzyme for hydrolyzing SM, with ceramide and phosphorylcholine as products. PLC is classified as a phosphoric acid diester hydrolase which catalyses the hydrolysis of glycerophospholipids to diacylglycerol and organic phosphate. The structure of SM is similar to phosphatidylcholine (PC), which is one of the preferred substrates of PLC. Both of them contain a phosphorylcholine and the sn-2 carbonyl group, which are the common substrate features for binding to *Bacillus cereus* PLC (Hansen et al., 1993). Enzymatic SM hydrolysis to ceramide and phosphorylcholine has been obtained with several preparations of PLC from bacteria, plants and some animal sources (Barnholz et al., 1966).

Since SM is a water-insoluble lipid, the enzymatic reaction can only be performed in an organic solvent–water system or an aqueous system containing a detergent (micellar system). Micellar systems have been broadly used for hydrolysis (Aalmo et al., 1984; Barnholz et al., 1966; Hanada et al., 2002), and micelles have been considered as superior substrates for catalysis by PLC from *B. cereus* (Little, 1977b). However, micellar systems are unsuitable for large-scale production systems since separation of product from the emulsifier and the requirement of high-speed stirring to prevent aggregate formation complicate the practical exploitation of the procedure. Furthermore, inclusion of detergents in the system could also disturb product analysis. Therefore, a water-saturated organic solvent system and a two-phase (water:organic solvent) system were compared for the hydrolysis reaction in the present study.

The catalytic activity towards SM was examined in several enzymes and several factors were examined to determine the optimal conditions for the ceramide pro-

duction. Finally, the hydrolysis reaction was optimized using response surface methodology (RSM) including the five factors selected.

2. Materials and methods

2.1. Materials

Standard chemicals were of the highest commercial purity available. Chicken egg yolk SM and bovine brain SM (99% purity) were purchased from Sigma–Aldrich Denmark A/S (Copenhagen, Denmark). Standard SM and ceramide from bovine brain for analysis were obtained from Avanti Polar lipids Inc. (Alabama, USA). The characteristics and abbreviations of the enzymes used for the study were listed in Table 1.

2.2. Sphingomyelin hydrolysis in water-saturated toluene

The reactor was a flat bottom headspace vial with a headspace aluminum crimp cap for sealing (Agilent Technologies Mfg GmbH, Waghäusel-Wiesental, Germany). Its radius was 1.1 cm, and its height was 4.6 cm. One hundred milligram SM was dissolved in 10 ml water-saturated toluene containing 150 μ l methanol to enhance the solubility. The solution was kept at -20°C until use. Buffer saturated toluene was prepared by mixing 0.1 M Tris–HCl buffer (pH 7.4) with toluene. One-milligram enzyme powder, 1.6 ml buffer saturated toluene and 0.4 ml SM solution were added to the reactor. The reactor was sealed after adding a stirrer. The reaction was started with the addition of the SM solution. Stirring speed was 450 rpm and

temperature was 37°C . A 50 μ l aliquots of reaction mixture were withdrawn at the desired intervals using a syringe (Hamilton Co., Reno, USA). The samples were stored at -20°C until analysis.

2.3. Sphingomyelin hydrolysis in two-phase system

A buffer mixture with a pH range from 2.0 to 12.0 was prepared and titrated with 0.4 M NaOH to pH 7.4 (Dawson et al., 1986). Enzymes were dissolved in the buffer solution in a final concentration of 250 U ml^{-1} , except for the SMase that was stored in buffer solution (125 U ml^{-1}). A 40 μ l enzyme solution, 1.76 ml toluene and 0.2 ml SM solution were added to the reactor to start the reaction at 37°C . A 50 μ l aliquots of reaction mixture in upper phase were withdrawn at the desired intervals. Other procedures were the same as described above.

To investigate the influence of certain ions on the catalytic activity, buffer solutions containing 25% ethanol, 0.15 M $(\text{NH}_4)_2\text{SO}_4$, 0.15 M NaCl and 0.01 M CaCl_2 were prepared individually, at pH 8.6. Enzyme was dissolved in these solutions with the final concentration of 250 U ml^{-1} . A 40 μ l enzyme solution was used for each hydrolysis. For optimization experiment, 1 ml reaction mixture (upper phase) was withdrawn and filtrated by a 0.45 μm HPLC filter (SupWare, Allerød, Denmark). The filtrated mixture was stored at -20°C prior to analysis.

2.4. Factor screening using response surface methodology

With the aim of simple factor screening, a fractional factorial resolution III design based on the principle of

Table 1
Activities, sources and abbreviations of the enzymes used

Name	Activity ^a	Source	Abbreviation
<i>B. cereus</i> PLC	100 ^b	Asahi-Kasei Co. (Tokyo, Japan)	BC-PLC(A)
<i>B. cereus</i> PLC (Type IV)	1202	Sigma–Aldrich Denmark A/S (Copenhagen, Denmark)	BC-PLC(IV)
<i>C. perfringens</i> PLC (Type I)	45	Sigma–Aldrich Denmark A/S (Copenhagen, Denmark)	CP-PLC(I)
<i>C. perfringens</i> PLC (Type IX)	66	Sigma–Aldrich Denmark A/S (Copenhagen, Denmark)	CP-PLC(IX)
<i>B. cereus</i> SMase	347	Sigma–Aldrich Denmark A/S (Copenhagen, Denmark)	SMase

^a Activity, units per protein (U mg^{-1}). One unit was defined as the amount of enzyme liberating 1.0 μmol of organic phosphate from the preferred substrate per minute under test condition.

^b Units per lyophilized powder (U mg^{-1}).

RSM was used to evaluate the impact of three factors: $(\text{NH}_4)_2\text{SO}_4$, NaCl and CaCl_2 . The type of factors was qualitative and seven experimental settings were generated. The only response was ceramide concentration (mg ml^{-1}) after 40 min reaction time.

2.5. Reaction optimization using response surface methodology

For optimization, a central composite design circumscribed with star distance 2 was used according to the principle of RSM. Using the five factors, 29 experimental settings were generated. The factors were enzyme amount [En, unit per reaction mixture (U ml^{-1})], water concentration (H_2O , %), ethanol

concentration (eth, %), reaction time (RT, min) and the ratio of hexane in ethylacetate (HR, %). The only response was ceramide concentration (mg ml^{-1}). The ethanol concentration was the volume percentage of ethanol in whole reaction mixture, and its range was set to be 0.3–2.7%. The ranges for the other factors were set based on the initial studies to: H_2O , 1.5–7.5%; En, 1–9 U ml^{-1} ; RT, 30–90 min; HR, 30–70%. The variables and the applied ranges are presented in Table 2.

2.6. Statistical analysis

The data were analyzed by means of RSM with software MODDE 6.0 (Umetrics, Umeå, Sweden).

Table 2

Experimental setup for the response surface methodology design and the responses obtained from the experiments and analysis^a

Experimental number	RT	En	H_2O	eth	HR	Cer. Con.
1	45	3	3	0.9	60	0.123
2	75	3	3	0.9	40	0.106
3	45	7	3	0.9	40	0.435
4	75	7	3	0.9	60	0.416
5	45	3	6	0.9	40	0.176
6	75	3	6	0.9	60	0.372
7	45	7	6	0.9	60	0.306
8	75	7	6	0.9	40	0.433
9	45	3	3	2.1	40	0.040
10	75	3	3	2.1	60	0.049
11	45	7	3	2.1	60	0.44
12	75	7	3	2.1	40	0.123
13	45	3	6	2.1	60	0.047
14 ^b	75	3	6	2.1	40	0.042
15	45	7	6	2.1	40	0.459
16	75	7	6	2.1	60	0.445
17	30	5	4.5	1.5	50	0.35
18	90	5	4.5	1.5	50	0.464
19	60	1	4.5	1.5	50	0.413
20 ^b	60	9	4.5	1.5	50	0.448
21	60	5	1.5	1.5	50	0.012
22	60	5	7.5	1.5	50	0.47
23	60	5	4.5	0.3	50	0.423
24	60	5	4.5	2.7	50	0.346
25	60	5	4.5	1.5	30	0.333
26	60	5	4.5	1.5	70	0.195
27	60	5	4.5	1.5	50	0.505
28	60	5	4.5	1.5	50	0.462
29	60	5	4.5	1.5	50	0.514

^a Abbreviation and unit: RT, reaction time (min); En, enzyme amount (U ml^{-1}); H_2O , water amount (%); eth, ethanol amount (%); HR, the hexane ratio of organic solvent (%); Cer. Con., ceramide concentration (mg ml^{-1}).

^b Outlier.

Second-order coefficients were generated by regression analysis with backward elimination. Response was first fitted to the factors by multiple regression. The quality of fit of the model was evaluated by the coefficients of determination (R^2) and the analysis of variances (ANOVA). The insignificant coefficients were eliminated stepwise after examining the coefficients and the model was finally refined. The quadratic response surface model was fitted to the following equation:

$$Y = \beta_0 + \sum_{i=1}^5 \beta_i x_i + \sum_{i=1}^5 \beta_{ii} x_i^2 + \sum_{i=1}^4 \sum_{j=i+1}^5 \beta_{ij} x_i x_j \quad (1)$$

where Y is the response variable, x_i the i th independent variable, β_0 the intercept, β_i the first-order model coefficient, β_{ii} the quadratic coefficient for the variable i and β_{ij} is the model coefficient for the interaction between factor i and j .

2.7. Analysis method

Sphingomyelin and ceramide concentrations were quantified using high performance thin-layer chromatography (HPTLC) and in situ densitometry, as earlier described (Müller et al., 2004). Briefly, the HPTLC-plate (Silica gel 60, E. Merck, Darmstadt, Germany) was pre-washed through development in chloroform:methanol (2:1 v/v) in a horizontal developing chamber (Camag, Muttentz, Switzerland), and the plate was activated at 120 °C for 30 min. Standards and samples were applied using DESAGA AS30 HPTLC Applicator (Sarstedt Ag & Co., Nümbrecht, Germany). Following equilibration and development with heptane:isopropanol:acetic acid (85:15:1) in the horizontal TLC chamber, the plate was dried and sprayed with charring reagent (10% CuSO₄ in 8% H₃PO₄) until soaked. Lipids were visualized by heating the plate at 160 °C for 6 min. The intensity of the spots was determined using DESAGA CD60 HPTLC Densitometer (Sarstedt Ag & Co.) at 390 nm in the absorbance/reflection mode. The lipid mass of each component was calculated with the software DESAGA ProQuant (1.03.200, Sarstedt Ag & Co.) using a standard curve run on the same plate. SM hydrolysis ratio was defined as the hydrolyzed SM (ceramide mass multiplied by the molecular weight ratio of SM to ceramide) divided by the initial SM amount.

3. Results and discussion

3.1. System evaluation

Micellar system has been widely used in organic biocatalysis but has some drawbacks such as complexity in recovery of product from the emulsifier and requirement of high-speed stirring to prevent clumping. In the present study, SM hydrolysis in water-saturated toluene and in two-phase (water:toluene) system were conducted and compared. Three enzymes, BC-PLC(A), BC-PLC(IV) and CP-PLC(I), were used to hydrolyze egg yolk SM. In water-saturated toluene, reaction time was 48 h, and enzyme amount was 1 mg (100, 180 and 9.9 units for BC-PLC(A), BC-PLC(IV) and CP-PLC(I), individually). In two-phase system, reaction time was 22 h, and the amount of every enzyme was 20 units.

Although the reaction conditions were not the same, it is evident that two-phase system was superior to the other system for the enzyme reaction (Table 3). In water-saturated toluene, the water concentration is 18.33 μmol ml⁻¹, which is adequate as a substrate for the hydrolysis reaction. The existence of a water phase enhanced the SM hydrolysis, indicating that water not only plays a role of nucleophile, but also contributes to the active state of the enzyme, as discussed before (Thrige et al., 1997; Hansen et al., 1993). PLC shows a marked preference for hydrolyzing substrates at a lipid–water interface (Little, 1977b), which could explain the enhanced activity of the enzyme. Therefore, the existence of water phase is an indispensable factor for the present catalysis reaction. The influence of water amount on the reaction was further investigated in later optimization experiment.

Table 3

The ratio of hydrolyzed SM to total SM (%) in the water-saturated toluene and the two-phase system^a

Enzyme	BC-PLC (A)	BC-PLC (IV)	CP-PLC (I)
Water-saturated toluene	4.0	1.1	7.7
Two-phase system	51.3	81.5	88.9

^a In water-saturated toluene, reaction time was 48 h and enzyme amount was 1 mg (100, 180 and 9.9 units for BC-PLC(A), BC-PLC(IV) and CP-PLC(I), respectively). In two-phase system, reaction time was 22 h and the amount of every enzyme was 20 units.

3.2. Enzyme screening

To examine the performance of different enzymes in the SM hydrolysis, five enzymes, BC-PLC(A), BC-PLC(IV), CP-PLC(I), CP-PLC(IX) and SMase were used to hydrolyze egg yolk SM in the two-phase system. A 80 μ l enzyme solution and 0.4 ml SM solution were added in the reactor. Due to different concentrations of the supplied enzyme, 10 units of SMase and 20 units of other enzymes were used. In accordance with the results in water-saturated toluene, CP-PLC(I) had the highest SM conversion, nearly 90% SM was degraded after 22 h (Fig. 2). BC-PLC(A) had the lowest conversion, about 50% was converted. The PLC used had the same enzyme activity measured as

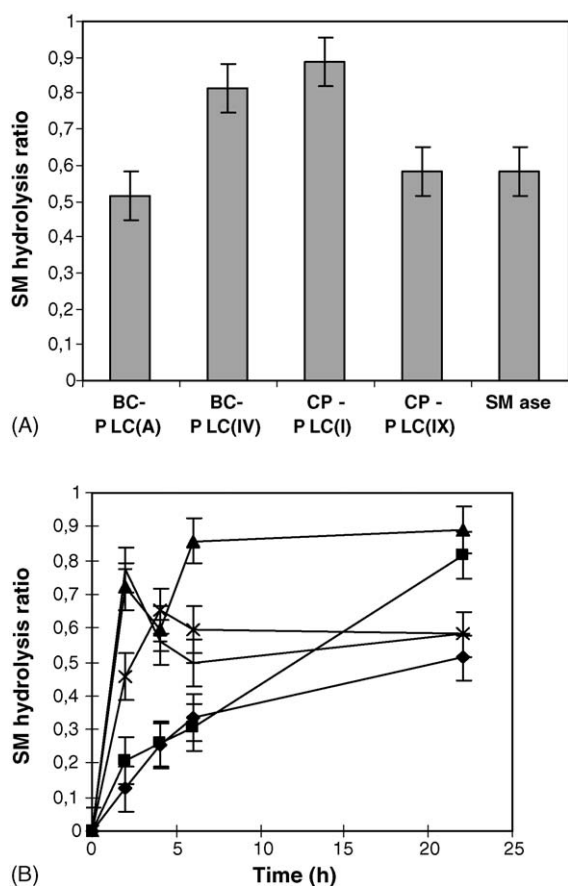


Fig. 2. Sphingomyelin hydrolysis with different enzymes in the two-phase system: (A) SM hydrolysis at 22 h; (B) the time course of hydrolysis. Legend: BC-PLC(A) (◆), BC-PLC(IV) (■), CP-PLC(I) (▲), CP-PLC(IX) (×) and SMase (—). Abbreviations see Table 1.

the PC hydrolysis. However, *Clostridium perfringens* PLC and *B. cereus* PLC can degrade SM at 35% and 1% of the rate of the PC hydrolysis, respectively (Krug and Kent, 1984; Waite, 1987). This big difference in substrate specificity implies higher activity of *C. perfringens* PLC towards the SM hydrolysis than that of *B. cereus* PLC, consistent with the results of the SM hydrolysis in the two systems. As expected, SMase had the highest hydrolytic activity, although it was used in half the amount of units compared with other enzymes, nearly 60% was degraded after 22 h. However, since SMase is much more expensive than the PLC, it is our intention to find a suitable PLC as a replacement. Therefore, *C. perfringens* PLC was selected in the current application.

3.3. Temperature and pH influence

Based on the results of the experiment above, CP-PLC(I) was selected for further investigation and optimization in the two-phase system. Temperature and pH are two basic properties of an enzyme. Different enzymes have different temperature optima and pH optima. The two optima may be even affected by the systems used. In this study, the chosen temperatures for evaluation were 22, 30, 37, 45 and 55 °C. After 22 h of reaction, the difference in SM conversion between the highest (37 °C) and the lowest (22 °C) was rather small (Fig. 3). Therefore, the enzyme has little dependence

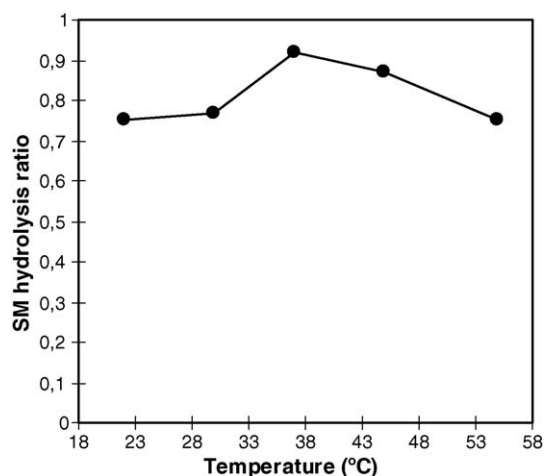


Fig. 3. Temperature influence on sphingomyelin hydrolysis using CP-PLC(I) as catalyst in the two-phase system for the reactions at 22 h. Abbreviations see Table 1.

on temperature. Normally, the effect of temperature on reaction rate can be described using the Arrhenius equation:

$$k = A \exp\left(\frac{-E_a}{RT}\right) \quad (2)$$

where k is the rate coefficient, A constant, E_a the activation energy, R the universal gas constant and T is the temperature (in degrees Kelvin). According to this equation, the reaction rate increases when the temperature rises. Nevertheless, the Arrhenius equation only applies when only one rate constant limits the rate in the entire temperature interval and when other factors such as thermal inactivation can be ignored. In the current two-phase system, the catalysis reaction occurred in the interface between water phase where enzyme was dissolved and organic solvent phase in which substrate remained. Hence other factors, for instance diffusion factor and the ratio of two phases, were possibly involved in the rate limiting issue, resulting in a reduced temperature effect. The highest SM conversion occurred at 37 °C; this value was applied in all further experiments.

pH influence was tested in a broad range from 3.5 to 9. After hydrolysis, ceramide could not be detected at pH below 4.5, although the SM content also was low (not shown). This could be explained by acidic hydrolysis of ceramide and SM at the low pH range. Fig. 4

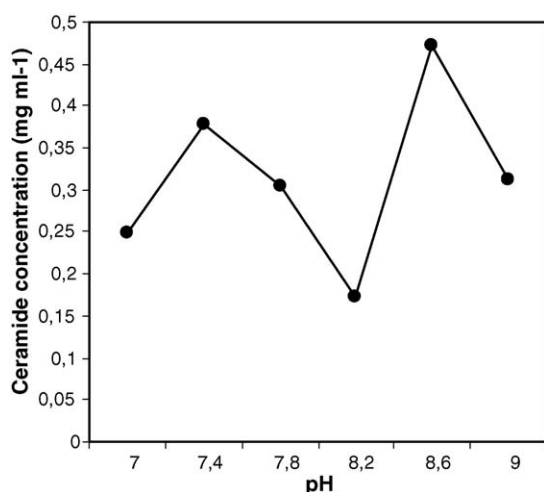


Fig. 4. pH influence on sphingomyelin hydrolysis using CP-PLC(I) as catalyst in the two-phase system for the reactions at 22 h. Abbreviations see Table 1.

shows the result of the reaction near the optimal pH area at 22 h. The SM hydrolysis had two peaks at pH 8.6 and 7.4, with the highest ceramide production at pH 8.6. This result was confirmed by repeated experiments. This phenomenon has not been reported, especially for the enzyme used. *C. perfringens* PLC contains two domains: an α -helical amino-terminal domain and a β -sheet-containing carboxy-terminal domain (Clark et al., 2003). Whilst the N-domain retains PC hydrolyzing activity of the PLC, the C-domain is thought to confer SMase activity on PLC. It has been suggested that C-domain facilitates PLC binding to the polar head groups of phospholipids, whereupon they are hydrolyzed by the catalytic N-domain (Clark et al., 2003). If the binding step and the subsequent catalytic step possess different favorable pH due to the relative functional groups staying in different domains, then the apparent pH plot will display the combination from these two pH influences. This could be the reason of two pH peaks in the hydrolysis. The other possible reason is that isomers or two enzymes that have similar substrate specificity probably exist in the PLC product.

3.4. Ions and ethanol influence on catalytic activity

Since ions and ethanol have been used in the PLC-catalyzed reactions (Aalmo et al., 1984; Susumu et

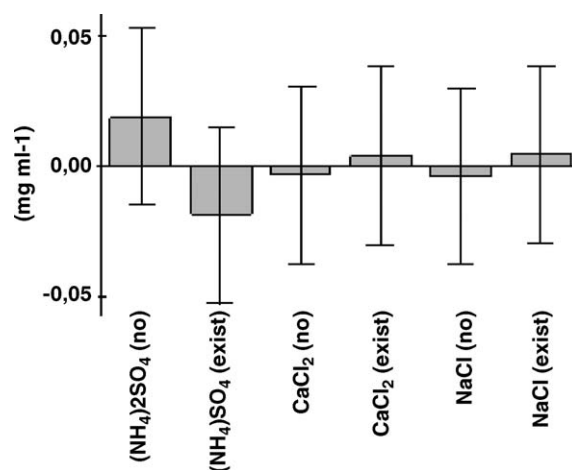


Fig. 5. The effect of additions of (NH₄)₂SO₄, CaCl₂ and NaCl on CP-PLC(I)-catalyzed hydrolysis reaction. The response is ceramide concentration (mg ml⁻¹). Abbreviations see Table 1.

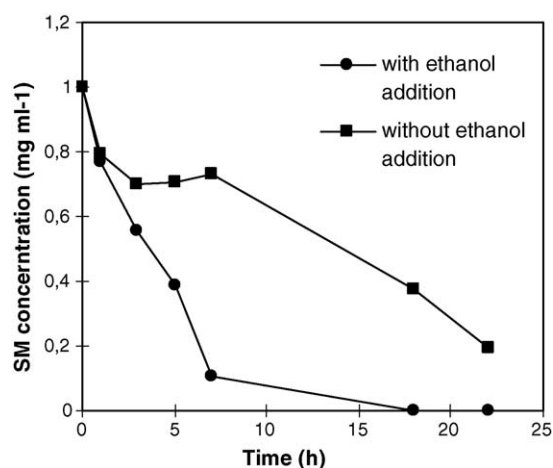


Fig. 6. Ethanol enhancement on sphingomyelin hydrolysis using CP-PLC(I) as catalyst in the two-phase system. The results are average values of duplicated experiments. Abbreviations see Table 1.

al., 1999; Krug et al., 1979), it is necessary to check the influence of these chemicals when SM is used as a substrate. The evaluation was conducted through a factor screening experimental design with the assistance of commercial software MODDE 6.0. After statistical analysis, the result showed that the addition of $(\text{NH}_4)_2\text{SO}_4$, NaCl and CaCl_2 had no significant effect on the hydrolysis (Fig. 5). In the enzyme assay,

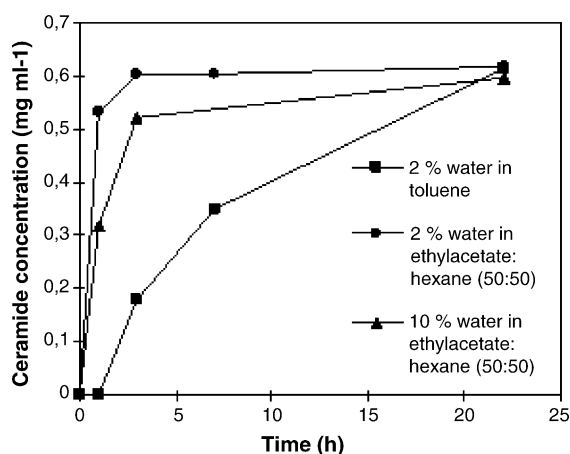


Fig. 7. Time courses of sphingomyelin hydrolysis using CP-PLC(I) as catalyst in different solvent systems. Abbreviations see Table 1.

no activity towards PC hydrolysis is obtained in the absence of a divalent cation, where the function of Ca^{2+} is inferentially to interact with substrate and provide a desired charge density (Krug and Kent, 1984). In the present study, protein content of the enzyme product from the company was 0.29 protein per solid (mg mg^{-1}). The above ions might have already been in the enzyme product, making the influence insignificant. This might be the reason for the discrepancy from the previous results. It could be true also, that the ion

Table 4

Regression coefficients describing the influence of different parameters on ceramide concentration (mg ml^{-1})^a

Variables	Coefficient	Standard error	P-value	Conf ^b
Constant	0.5000	0.0313	5.83×10^{-9}	0.0689
RT	0.0308	0.0124	0.030	0.0272
En	0.0756	0.0126	8.92×10^{-5}	0.0277
H ₂ O	0.0835	0.0123	2.89×10^{-5}	0.0270
eth	−0.0125	0.0123	0.331	0.0270
HR	−0.0235	0.0124	0.084	0.0272
RT·RT	−0.0280	0.0112	0.030	0.0247
H ₂ O·H ₂ O	−0.0656	0.0112	1.10×10^{-4}	0.0247
eth·eth	−0.0302	0.0112	0.021	0.0247
HR·HR	−0.0570	0.0112	3.52×10^{-4}	0.0247
RT·En	−0.0560	0.0132	1.41×10^{-3}	0.0291
RT·H ₂ O	0.0696	0.0146	5.73×10^{-4}	0.0321
En·H ₂ O	−0.0414	0.0132	9.60×10^{-3}	0.0291
En·HR	0.0303	0.0132	0.043	0.0291
H ₂ O·eth	0.0423	0.0146	0.014	0.0321
H ₂ O·HR	−0.0486	0.0146	6.62×10^{-3}	0.0321

^a For abbreviation see Table 2 and the text.

^b Conf, the 95% confidence interval (\pm) on the coefficient value.

influence on the catalytic activity is not crucial in such a system.

However, ethanol addition to the system markedly enhanced the hydrolysis by CP-PLC(I) (Fig. 6). By adding 25% ethanol to the enzyme solution, the hydrolysis rate was considerably enhanced. This phenomenon has been reported for many phospholipases (Miller et al., 1993; Kisel et al., 2001). There are mainly two possible reasons in the present case. One is that ethanol addition does increase the solubility of SM and ceramide, hereby enhance the rate of diffusion to and from the active site. Water and the organic solvent are immiscible, and ethanol can play a role for better mass transfer in their mixture. The accelerating product release can definitely enhance hydrolysis reaction. The

other reason is that ethanol can change the properties of PLC. Twenty-five percent ethanol could push the exposure of histidine groups in *B. cereus* PLC (Little, 1977a), where the histidine groups in the active site play a crucial role in catalytic properties. The effect of ethanol amount on the reaction will be checked in the optimization experiments.

3.5. Solvent test

Toluene is toxic and flammable, resulting in much caution in applications. Ethylacetate and hexane are also broadly used as organic solvent with less risk than toluene. From Snyder polarity index, the polarities of ethylacetate and hexane are 4.4 and 0, respectively. In order to imitate the polarity of toluene, which is 2.4, we decided to use ethylacetate:hexane (50:50) instead of toluene. Different water content from 2% to 10% based on the solvent mixture was also tested. Fig. 7 shows the results of the test with CP-PLC(I) as catalyst. The use of ethylacetate:hexane (50:50) as the medium markedly boosted the SM hydrolysis in the system, indicating the solvent dependence of the reaction. The solubility of ceramide may be increased by the formation of hydrogen bond with the oxygen atom in ethylacetate. As a result, ceramide may be easier to release from PLC, so as to improve the hydrolysis. Water solubility in ethylacetate is higher than in toluene, implying that the

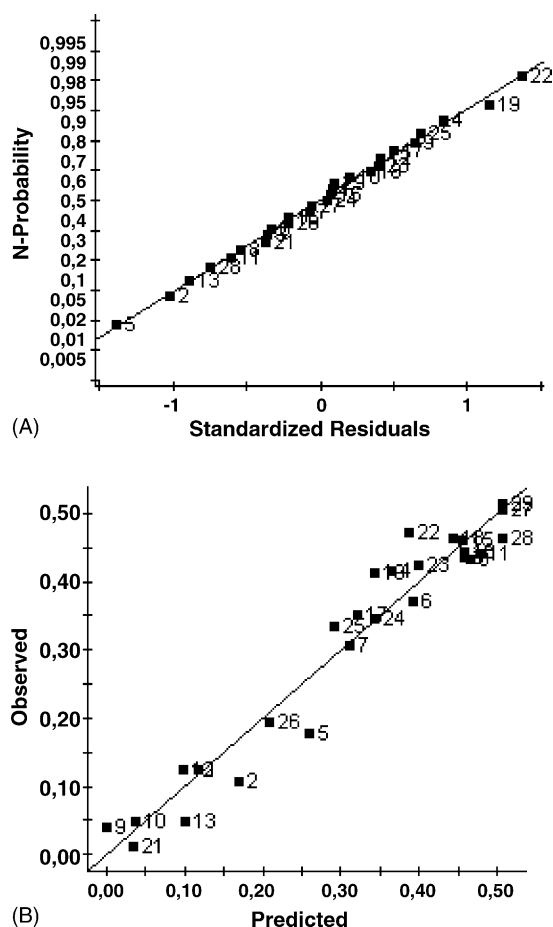


Fig. 8. The normal probability plot of residuals (A) and relationship plot between the observed and predicted ceramide concentration (B). The numbers inside the graph represent the experimental numbers.

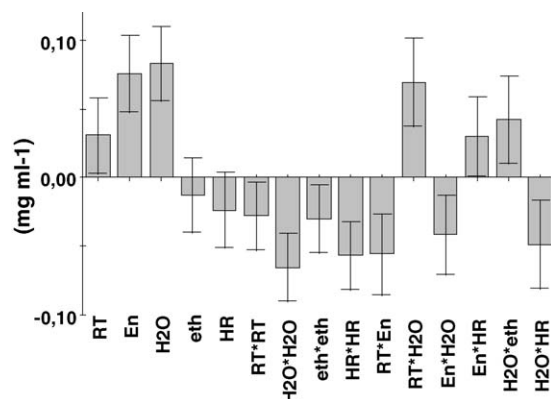


Fig. 9. Summary of effects on sphingomyelin hydrolysis using CP-PLC(I) as catalyst in the two-phase system. Five factors, enzyme amount (En), water amount (H₂O), ethanol amount (eth), reaction time (RT) and the hexane ratio of organic solvent (HR), were selected for optimization. The only response is ceramide concentration (mg ml⁻¹). Abbreviations see Table 1.

access of SM to and the dispersion of ceramide in the active site of PLC can be improved by the use of the new solvent mixture. This is the other possible reason for the increase of the hydrolysis. Since the reaction had a better performance in ethylacetate:hexane (50:50), toluene was replaced with the solvent mixture in the remaining experiment.

3.6. Optimization using response surface methodology

From the above study, we can see that a potential system for the SM hydrolysis has been created, includ-

ing a suitable enzyme [CP-PLC(I)], a better solvent system [ethylacetate:hexane (50:50)], no ions necessary and a suitable temperature (37 °C) and pH (8.6). However, to optimize the system, we need to know the amount of water, the amount of ethanol, and the ratio of the two organic solvents, as well as their interactions with the length of reaction time and the amount of enzyme. These issues can be better solved through an optimization operation assisted by RSM. RSM is an effective statistical technique for the optimization of complicated systems, which enables the evaluation of effects from multiple parameters, alone or in combination, on response variables. The main advantage of

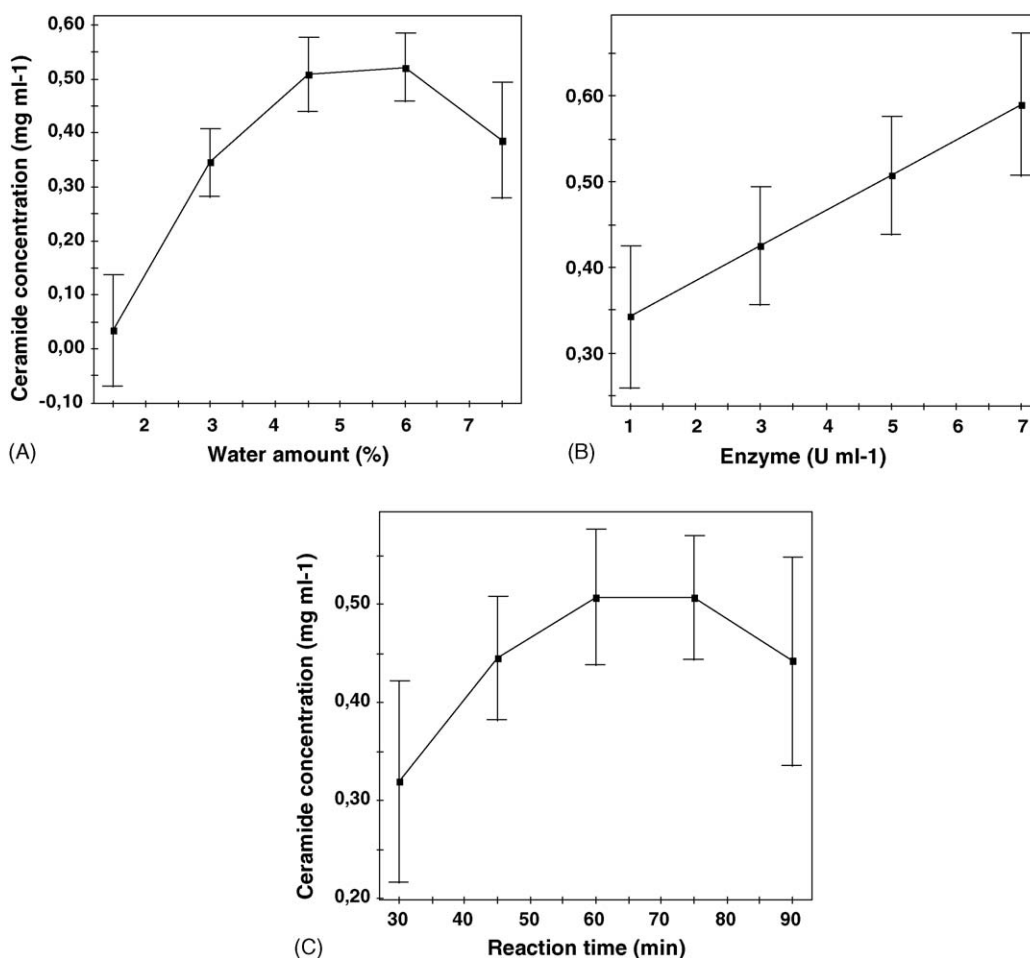


Fig. 10. Main effect plots for CP-PLC(I)-catalyzed hydrolysis reaction in the two-phase system: (A) water amount; (B) enzyme amount; (C) reaction time. The main effect plot displays the predicted change in the response when the factor varies from its low to its high level, all other factors in the design being set on their average (eth, 1.5%; H₂O, 4.5%; En, 5 U ml⁻¹; RT, 60 min; HR, 50%). Abbreviations see Table 1 and Fig. 9.

RSM is the reduced number of experiments needed to provide sufficient information for statistically acceptable results (Xu et al., 1998). The five parameters and their ranges in Table 2 were decided based on previous studies. The responses were first fitted to the parameters. The best fitting quadratic model was determined by regression and backward elimination. The model coefficients and *P* values for the regression variables are given in Table 4. Most *P* values of the coefficient were below 0.05 after the model was refined. The coefficient of determination (R^2), which is a measure of fit, i.e. how well the model fits the data, was 0.94. The reproducibility coefficient was 0.97. The normal probability plot of residuals had all the points in a good

linear relationship within a narrow range of standardized residues (-1.5 to 1.5) (Fig. 8A). The observed and predicted values were also sufficiently correlated (Fig. 8B). This pointed out that the model generally represented the actual relationships between the response and the reaction parameters within the ranges selected.

Fig. 9 is the summary of effects on the reaction based on the model defined. Water and enzyme amount had the most significant influence on the hydrolysis, followed by reaction time. Ethanol amount and the ratio of two organic solvents are not significant, indicating that the variation of the two parameters in the selected ranges does not significantly affect

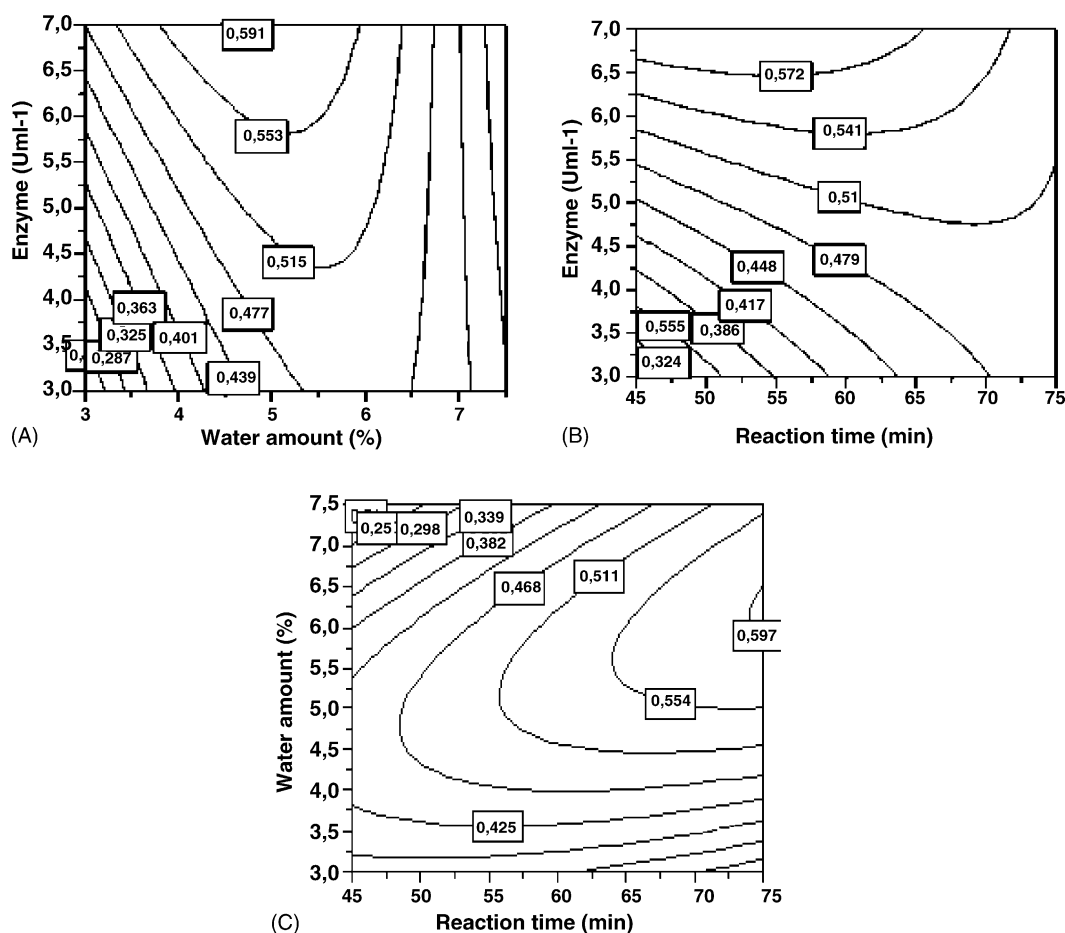


Fig. 11. The contour plots for CP-PLC(I)-catalyzed sphingomyelin hydrolysis reaction in the two-phase system: (A) enzyme amount vs. water amount; (B) enzyme amount vs. reaction time; (C) water amount vs. reaction time. The numbers inside the contour plots indicate the ceramide concentration (mg ml⁻¹). Abbreviations see Table 1.

the system. This may imply that the function of the PLC may play the main reason since the solubility or diffusion should be affected by these two parameters. In order to have a close look of the three significant parameters, the main effect of each parameter is given in Fig. 10. The consequence of water amount was discussed before, and the present result proved its importance again. Obviously, too much water is also not good (Fig. 10A). Enzyme amount exhibited a linear relationship, meaning that more enzyme amount, more ceramide will be produced when all other factors in the design were set on their average. Reaction time shows big error bar, but a too long reaction time is not recommended. The reason could be that ceramide formed can go further hydrolysis.

The relationships and interactions between the main effects could be illustrated by contour plots (Fig. 11) when other parameters in the design were set on their average (eth, 1.5%; H₂O, 4.5%; En, 5 U ml⁻¹; RT, 60 min; HR, 50%). In the contour plot between enzyme amount and water amount (Fig. 11A), the optimal conditions can be observed with enzyme amount 7 U ml⁻¹ and water amount 5%. There was a chine in the intersection between two inclined surfaces, revealing the existence of an optimal linear relationship between enzyme amount and water amount, where the best SM hydrolysis would lie. This linear relationship is useful for calculation to decide the rate of these two factors or PLC concentration in enzyme solution. In the contour plot between reaction time and enzyme amount (Fig. 11B), it is shown that longer reaction time enhanced the hydrolysis at low enzyme amount. However, at high enzyme amount, the ceramide concentration was decreased by longer time. Hence, altered enzyme amount also change the optimal reaction time.

In contour plots, there are several optimal combinations available to obtain the highest ceramide concentration. This gives the possibility to select suitable conditions flexibly, depending on the particular preference of the manufacturer. Through iterative calculation with the target of maximum ceramide concentration, the results recommend the general optimal conditions as 75 min reaction time, 3 U ml⁻¹ enzyme amount, 6% water amount, 1.8% ethanol amount and 46% hexane in organic solvent. Under these conditions, ceramide concentration should be 0.68 mg ml⁻¹ according to the model prediction, where the yield is 88%.

In summary, present study was the first time to carry on the systematic research focus on the enzymatic production of ceramide from SM hydrolysis. It was verified that SM modification gives a feasible approach to the potential production of ceramide. After system evaluation and optimization of several important factors, the performance of the hydrolysis was dramatically improved. Consequently, some results obtained from the study provided valuable information for further development and applications at industrial levels.

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PAPER II

Long Zhang, Lars I. Hellgren and Xuebing Xu (2007) Immobilization of phospholipase C for the production of ceramide from sphingomyelin hydrolysis, *Journal of the American Oil Chemists Society*, 84: 237-247

Immobilization of Phospholipase C for the Production of Ceramide from Sphingomyelin Hydrolysis

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Abstract The immobilization of *Clostridium perfringens* phospholipase C was studied for the first time and the catalytic properties of the immobilized enzyme were investigated for the hydrolysis of sphingomyelin to produce ceramide. Ceramide is of great commercial value in the cosmetic and pharmaceutical industries for use in, for example, hair and skin care products, owing to its major role in maintaining the water-retaining properties of the epidermis. The feasibility of enzymatic production of ceramide through hydrolysis of sphingomyelin has previously been proven. In order to improve the reusability of the enzyme, the present study focused on the immobilization of phospholipase C in the production of ceramide from sphingomyelin. By screening nine different carriers, we found that the enzyme immobilized on Lewatit had the highest catalytic activity towards sphingomyelin hydrolysis. Prewetting Lewatit with ethanol led to higher enzyme fixation on the carrier, but the activity of the enzyme was decreased. Increasing the initial enzyme concentration resulted in more enzyme adsorption on the carrier, where the specific activity was increased. Through optimization of the reaction using the immobilized enzyme, the optimal temperature was around 46 °C and the optimal water volume was 3.5%. The reaction had little dependence on pH. After seven recycles, immobilized enzyme retained around 70% of

the initial activity. Immobilized enzyme was deactivated irregularly when stored at room temperature, but followed first-order deactivation when stored at 40 °C.

Keywords Carrier · Ceramide · Hydrolysis · Immobilization · Optimization · Phospholipase C · Response surface methodology · Sphingomyelin

Introduction

Ceramide (Fig. 1a), the N-acylated derivative of sphingosine, is the key intermediate in the biosynthesis of all complex sphingolipids. Owing to its major role in maintaining the water-retaining properties of the epidermis [1–3], ceramide is of great commercial value in the cosmetic and pharmaceutical industries for use in hair and skin care products, for example. Many ceramide-containing products have already been introduced to the cosmetic market, and the effect of the application of ceramide is excellent. It is claimed that these products dramatically increase the skin's hydration level, repair the cutaneous barrier, prevent vital moisture loss, and contribute to reducing dry flaky skin and aged appearance. However, chemical synthesis of ceramide is a costly and time-consuming process for industrial applications; therefore, the development of alternative cost-efficient and high-yield production methods is of substantial interest.

Sphingomyelin (SM) is a ubiquitous component of animal cell membranes and it is one of the major phospholipids in bovine milk. In SM, the ceramide part of the molecule is bound through a phosphodiester bridge to a choline moiety (Fig. 1b). Ceramide production from SM hydrolysis has already been de-

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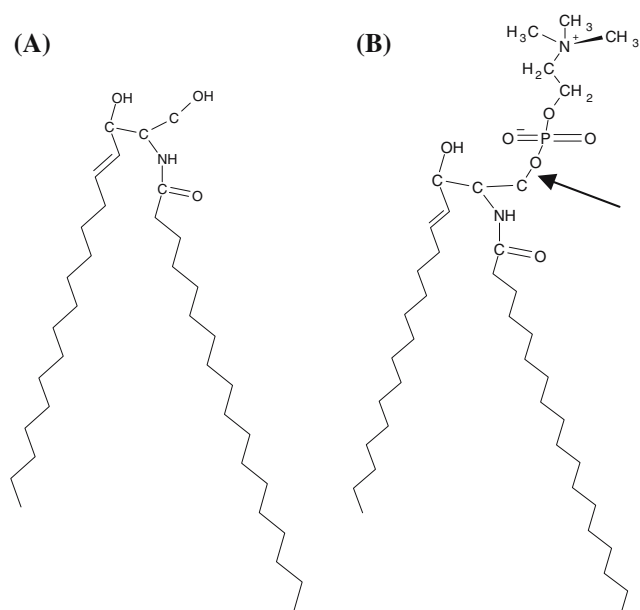


Fig. 1 The structures of ceramide (**a**) and sphingomyelin (**SM**) (**b**). The arrow indicates the cleaved bond in SM for producing ceramide

scribed in a few patents [2, 4]. Moreover, systematic investigation and optimization of the enzymatic production of ceramide from SM modification has been conducted in single-batch reactions [5]. The reaction system has been improved, and phospholipase C (PLC, EC 3.1.4.3) from *Clostridium perfringens* shows high activity towards the hydrolysis reaction. It has been verified that SM modification gives a feasible approach to the potential production of ceramide. However, the price of PLC is high, and this would hinder further development and applications at industrial levels; thus, it is necessary to consider the reuse of the enzyme, which would significantly reduce enzyme cost.

Enzyme immobilization is a process to attach an enzyme to an insoluble matrix, while its catalytic activity is still retained. Through immobilization, the enzyme can be physically separated from the bulk reaction medium and, at the same time, is permeable to reactant and product molecules [6]. The advantages of enzyme immobilization can be summarized as follows:

1. The enzyme can be reused and utilized in a more efficient way.
2. Enzyme stability is often increased by immobilization [7].
3. An immobilized enzyme (IE) sometimes offers better process possibilities than a soluble enzyme.
4. Enzyme immobilization avoids the contamination of the products with residual enzymatic activity.

The observed properties of the enzyme are invariably changed by immobilization. The type and the magnitude of these changes depend on the enzyme and the immobilization method used [7]. Many studies of enzyme immobilization have focused on the application of lipase [6]. However, the immobilization of PLC to catalyze SM hydrolysis has not been emphasized and systematically studied.

The aim of present study was to investigate the feasibility of PLC immobilization in the production of ceramide from SM hydrolysis. In order to find the best immobilization process, nine different carriers were screened, and the effects of ethanol as well as the initial enzyme concentration were evaluated. The hydrolysis reaction of IE was optimized for several important factors, individually and together with the assistance of response surface methodology (RSM). Finally the operational and storage stabilities of IE were examined under the optimal conditions.

Materials and Methods

Materials

Standard chemicals were of the highest commercial purity available. Bovine brain SM (approximately 99%), standard ceramide (99% or more) from bovine brain, and *Clostridium perfringens* PLC (type I) were purchased from Sigma-Aldrich Denmark (Copenhagen, Denmark). The carriers used for the present study are described in Table 1.

Immobilization Process

The carrier (350 mg) was conditioned in 6 ml 96% ethanol for 20 min, followed by washing twice with 6 ml distilled water and drying in a fume hood overnight. The carrier (200 mg) was suspended in 2 ml enzyme solution [1.14 mg protein/ml 0.01 M tris(hydroxymethyl)aminomethane (Tris)–HCl buffer, pH 8.6]. The mixture was shaken on a tilting mixer shaker (J.P. Selecta, Barcelona, Spain) for 17 h. The incubation mixture was centrifuged at 3,000 rpm in 1 min. A 0.5-ml aliquot of the clear solution was taken out for analysis of the protein concentration according to the Lowry method [8] using bovine serum albumin as the standard. The solids in the mixture were obtained through vacuum filtration and were washed three times with 5 ml buffer solution. IE was dried through 5-min suction with a vacuum pump followed by 5-h in the fume hood. In the study of the effects of ethanol on the

Table 1 The carriers and their characteristics

Carrier name	Abbreviation	Company	General description
Amberlite® XAD7	XAD	Sigma-Aldrich Chemie, Steinheim, Germany	Nonionic weakly polar macroreticular resin (matrix, acyclic ester). Particle size, 0.25–0.84 mm (wet)
Supelite™ DAX-8	DAX	Supelco, Bellefonte, USA	Resin with moderate polarity (matrix, acrylic ester). Particle size, 0.25–0.42 mm
Celite 545	Cel	BDH, Poole, UK	Kieselguhr soda ash flux calcined. Particle size, 0.02–0.1 mm
Dowex® 50WX4	Dow	Dow Chemical Company, Midland, USA	Cation-exchange resin (matrix, styrene–divinylbenzene). Functional group, sulfonic acid. Particle size, 0.15–0.30 mm
Lewatit VP OC 1600	Lew	Bayer, Leverkusen, Germany	Divinylbenzene cross-linked polymer (matrix, methacrylate). Particle size, 0.315–1 mm
Duolite A568	Duo	Rohm and Haas, Chauny, France	Polymerized phenol–formaldehyde ion-exchange resin
Sepabeads FP-DA	FP	Resindion, Binasco, Italy	Polymethacrylate (active group, diethylamino). Pore size, >0.1 µm
Sepabeads FP-DA1	FP1	Resindion, Binasco, Italy	Polymethacrylate (active group, diethylamino). Pore size, >0.03 µm
Accurel EP 100	Acc	Akzo, Obernburg, Germany	Macroporous polypropylene support

immobilization process, the same procedure was followed, but 0.15 ml ethanol was added to 200 mg carrier before the carrier was mixed with 2 ml enzyme solution.

Activity Test for Soluble Enzyme

The reaction conditions for the soluble enzyme were as described in a previous study [5]. Briefly, the enzyme was dissolved in 0.01 M Tris–HCl buffer (pH 8.6) containing 25% ethanol, with a final enzyme concentration of 0.219 mg/ml. SM solution (0.1 ml of 20 mg/ml) in chloroform–methanol (2:1) was added to the reactor, and was dried under nitrogen. Then, 1.88 ml ethyl acetate–hexane (50:50) and 0.12 ml enzyme solution were added to the reactor. The reaction was started with the addition of the enzyme solution. The reaction was performed in a sealed reactor at 37 °C using a magnetic stirrer at 450 rpm. Aliquots of 50 µl of the reaction mixture were withdrawn at the desired time intervals using a syringe (Hamilton, Reno, USA). The samples were stored at –20 °C until they were analyzed.

Activity Test for Immobilized Enzyme

Dried IE (50 mg) was put into the reactor, instead of the enzyme solution. Buffer solution containing 25% ethanol (0.12 ml) and 1.88 ml ethyl acetate–hexane (50:50) was added to the reactor. If the reaction mixture was cloudy (depending on the type of carrier), the samples were centrifuged at 3,000 rpm for 2 min prior to sampling. Other procedures were as described above. For the carrier-screening study, 150 mg IE was used in the activity test.

Operational Stability Study

For the soluble enzyme, the lower phase (water phase) was transferred from the last reaction mixture to the new reaction bottle, in the interval between reactions. Then, the new reaction started without further addition of the enzyme. In the case of IE, the supernatant from the last reaction was discarded. Then, IE was washed with 2 ml ethyl acetate–hexane (50:50) and buffer solution containing 25% ethanol, respectively. The wet IE was transferred to a new batch without addition of buffer. The reaction time was 20 min for both the soluble enzyme and the IE. Other procedures were as described above.

Immobilized Enzyme Reaction Optimization Using Response Surface Methodology

For optimization, a central composite design circumscribed with star distance 1.682 was used according to the principle of RSM. Using the three factors, we generated 17 experimental settings. The factors were temperature, water volume and pH. The only response was the initial reaction rate. The water volume was the volume of buffer solution containing 25% ethanol added, and its range was set to be 35.9–204.1 µl. The ranges for other factors were set to 21.9–52.1 °C and pH 7.53–8.87. The variables and the ranges applied are presented in Table 2.

Statistical Analysis

The data were analyzed by means of RSM with the software MODDE 6.0 (Umetrics, Umeå, Sweden). Second-order coefficients were generated by regression

Table 2 Experimental setup for the response surface design and the responses obtained from the experiments and analysis

Exp no.	Temperature (°C)	H ₂ O (μl)	pH	Initial reaction rate (μg/min)
1	28	70	7.8	14.19
2	46	70	7.8	48.25
3	28	170	7.8	18.50
4	46	170	7.8	17.20
5	28	70	8.6	13.06
6	46	70	8.6	59.80
7	28	170	8.6	15.57
8	46	170	8.6	16.78
9	21.9	120	8.2	8.96
10	52.1	120	8.2	27.70
11 ^a	37	35.9	8.2	15.28
12	37	204.1	8.2	17.49
13	37	120	7.53	31.78
14	37	120	8.87	34.65
15	37	120	8.2	27.52
16	37	120	8.2	32.87
17	37	120	8.2	23.71

^a Outlier

analysis with backward elimination. The response was first fitted to the factors by multiple regression. The quality of the fit of the model was evaluated by the coefficients of determination (R²) and the analysis of variances (ANOVA). The insignificant coefficients were eliminated stepwise after examining the coefficients and the model was finally refined. The quadratic response surface model was fitted to the following equation:

$$Y = \beta_0 + \sum_{i=1}^3 \beta_i x_i + \sum_{i=1}^3 \beta_{ii} x_i^2 + \sum_{i=1}^2 \sum_{j=i+1}^3 \beta_{ij} x_i x_j, \quad (1)$$

where Y is the response variable, x_i the i th independent variable, β_0 the intercept, β_i the first-order model coefficient, β_{ii} the quadratic coefficient for the variable i , and β_{ij} the model coefficient for the interaction between factor i and j .

Analysis Method

Ceramide concentrations were quantified using high-performance thin-layer chromatography (HPTLC) and in situ densitometry, essentially as described earlier [9]. Briefly, the HPTLC plate (Silica gel 60, E. Merck, Darmstadt, Germany) was prewashed through development in chloroform–methanol (2:1 by volume) in a horizontal developing chamber (Camag, Muttens, Switzerland), and the plate was activated at 120 °C for 30 min. Standards and samples were applied using a DESAGA AS30 HPTLC applicator (Sarstedt,

Nümbrecht, Germany). Following equilibration and development with heptane–2-propanol–acetic acid (85:15:1) in the chamber, the plate was dried and sprayed with charring reagent (10% CuSO₄ in 8% H₃PO₄) until it was soaked. Lipids were visualized by heating the plate at 160 °C for 6 min. The intensity of the spots was determined using a DESAGA CD60 HPTLC densitometer (Sarstedt) at 390 nm in the absorbance/reflection mode. The lipid mass of each component was calculated with the software DESAGA ProQuant (version 1.03.200, Sarstedt) using a standard curve run on the same plate. The apparent activity was defined as the initial reaction rate divided by the amount of IE. The specific activity was defined as the initial reaction rate divided by the amount of protein. The fixation level was the percentage of the immobilized protein (estimated through subtracting the protein concentration remaining in the supernatant from the initial protein concentration). The degree of hydrolysis was the percentage of the hydrolyzed SM (ceramide mass multiplied by the molecular weight ratio of SM to ceramide).

Results and Discussion

Carrier Screening

Immobilization can make enzymes reusable and offer both better process possibilities and better process efficiency. There are many factors that can influence the process of enzyme immobilization. Firstly, the type of carrier was studied for optimization of the process. Nine different carriers were used for enzyme immobilization. Their performances were compared on the fixation level (Fig. 2a). The highest fixation level was found for Amberlite® XAD7 (XAD). Based on the same amount of IE, their activities were measured and compared (Fig. 2b). The highest activity was shown by the enzyme immobilized on Lewatit VP OC 1600 (Lew), and the degree of hydrolysis was 98% at 64 h. To enhance the activity of PLC immobilized on XAD, the buffer volume was increased from 120 to 400 μl in the hydrolysis reaction of IE. Adding more buffer increased the ceramide concentration from 0.05 to 0.2 mg/ml after 64-h reaction; however, its activity was still low compared with that of Lew-based products. Therefore, the following work focused on the study of immobilization using Lew as the carrier.

Most of the carriers in the current experiment are commonly used as supports in immobilization processes for lipases and phospholipases. As seen in Ta-

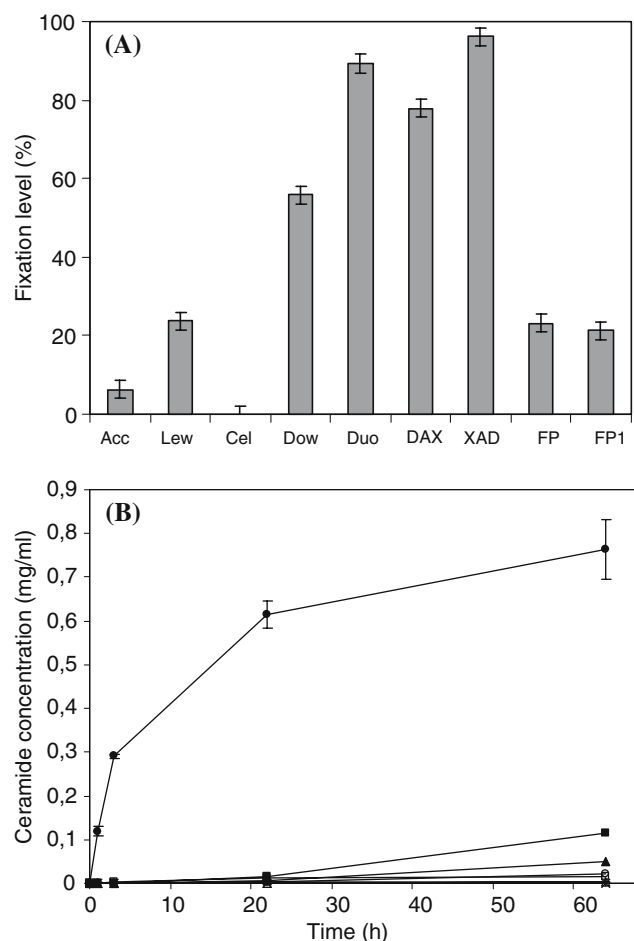


Fig. 2 Comparison of different carriers. The whole name and details of the nine carriers are given in Table 1. **a** Fixation levels of different carriers. **b** SM hydrolysis by differently immobilized phospholipase C based on the same amount of immobilized enzyme (IE). The error bars are the standard deviation calculated from repeat experiments using Lew. Open diamonds Acc, filled diamonds Lew, open triangles Cel, crosses Dow, horizontal bars Duo, filled squares DAX, filled triangles XAD, (pluses) FP, open squares FP1

ble 1, their chemical compositions, geometric characteristics, and immobilization principles are diverse, and these characteristics play a crucial role in the apparent catalytic activity of IE. Lew, a divinylbenzene cross-linked polymer (matrix, methacrylate), is hydrophobic. When mixed with the enzyme solution, Lew floated to the top of the solution. With the exception of Accurel EP 100, the other carriers were suspended in the solution. This is probably the explanation for the lower fixation levels in Lew. Though many factors, such as skeletal density, particle size, and porosity, can affect the activity of IE, the high activity of the enzyme immobilized on Lew is probably due to the hydrophobic properties of Lew. Examining the steric view of PLC from *Clostridium perfringens*, we can see that the

amino acids involved in catalysis in the active site are hydrophilic [10]. If the carrier is also hydrophilic, there is a large risk of the carrier interacting with the active site of the enzyme. Consequently, once the active site of PLC is blocked by this contact during the immobilization process, the activity of IE will be reduced; therefore, the performance of the IE reaction is highly sensitive to the properties of the carrier, especially its interaction with the amino acid residues in the active site. Furthermore, since both the substrate and the product of the reaction are hydrophobic molecules, the hydrophobic surroundings of the enzyme in the Lew particle will enhance substrate and product transfer to and from the active site, compared with the transfer in the presence of with hydrophilic particles.

Following immobilization, IE was washed five times, and the protein concentration of the effluents (3 ml each time) was measured. After washing twice, we detected no protein in the effluents, indicating that soluble enzyme had no effect on the analysis of the activity of IE. Although the enzyme immobilized on Lew had the highest activity, the specific activity of the IE, $533.9 \pm 10.1 \mu\text{g}/\text{mg}$ protein/min (optimal condition), was still low compared with that of the soluble enzyme, $2347.8 \pm 125.2 \mu\text{g}/\text{mg}$ protein/min, in the present reaction system. The reduction in catalytic activity of the enzyme after immobilization may have different causes, such as partial denaturation, change of enzyme kinetics, partition effects, and mass-transport limitations [11].

Effect of Ethanol on the Immobilization Process

Since Lew did not mix well with the enzyme solution during immobilization, it would be desirable to find methods to enhance mixing and thereby improve the fixation level of PLC on the carrier. Wetting Lew with ethanol prior to immobilization was an efficient method of increasing the fixation level from 25 to 74% (Table 3). Wetting with ethanol caused the carriers to swell (visual observation), which increased the pore size of the particles and probably improved the penetration of water through the surface. After ethanol treatment, the particles were mixed well with the solution, which probably explains the enhanced adsorption of PLC on the carrier.

However, the specific activity of IE was drastically decreased by prewetting Lew with ethanol. Actually, even the apparent activity of IE was reduced, despite the larger mass of the enzyme immobilized on the carrier (Table 3). Normally, the more enzyme used in the reaction, the higher the reaction rate. The strong

Table 3 The influence of prewetting Lew with ethanol on the immobilization of the enzyme

Procedure (before immobilization)	Fixation level (%)	Protein amount in IE (mg/g)	Apparent activity ($\mu\text{g/g IE/min}$)	Specific activity ($\mu\text{g/mg protein/min}$)
Prewetting Lew with 150 μl ethanol	75.0 ± 0.9	8.6 ± 0.1	20.6 ± 2.6	2.4 ± 0.3
Soaking Lew with ethanol, and washing ethanol out	81.8 ± 0.4	9.3 ± 0.1	72.5 ± 5.6	7.8 ± 0.6
No ethanol treatment	23.8 ± 2.3	2.7 ± 0.3	139.6 ± 2.2	51.7 ± 0.8

IE immobilized enzyme

inactivation induced by prewetting Lew with ethanol is possibly due to the following:

1. At low loading levels, PLC molecules would have less steric restriction, leading to a more favorable special arrangement for enzyme–substrate complex formation [12]. At high loadings, many active sites of PLC will be blocked and only a fraction of the available PLC would be involved in the catalytic reaction. A higher enzymatic load would increase the limitation of substrate diffusion and therefore decrease enzyme efficiency [12].
2. Ethanol probably has some inhibitive effect on the immobilized PLC. To test this effect, the carrier were soaked in 1 ml ethanol and washed with 4 ml buffer solution three times before immobilization. When the ethanol had been washed out, the fixation level of the enzyme also increased (Table 3); however, the specific activity of the enzyme decreased in a similar manner. As a result, the inhibitive effect of ethanol on PLC activity was not shown. Indeed, the addition of ethanol enhanced SM hydrolysis using soluble PLC from *Clostridium perfringens* in a previous study [5].
3. The carrier was swelled by wetting with ethanol, which increased the pore size of the particles. Consequently, the accessible surface area of the carrier, defined as the fraction of the total surface area the enzyme can approach during immobilization, was increased. Usually, the larger the accessible surface area, the greater the contact between the enzyme and the carrier, and a higher extent of inactivation can be expected, even though the chemical characteristics of the carrier and its affinity for the enzyme are also important [13].

Higher specific activity of IE occurred without ethanol treatment on the carrier, where the fixation level of the enzyme was low. If this characteristic is examined from an engineering point of view, two possible paths can be investigated for potential efficiency gains:

1. Using a larger amount of the carrier while keeping other parameters constant. Hereby, the fixation level will be increased since there are more absorption sites for the enzyme.

2. Reusing the enzyme solution. Since only 25% of the enzyme was immobilized on the carrier, 75% would be left in the solution.

To reduce the total enzyme cost, it is necessary to recycle the supernatant if the activity of the enzyme is not lost during the immobilization process. In the recycling step, the volume of the solution, the enzyme concentration, and the amount of the carrier can be adjusted to fulfill the specific interest.

Effect of Initial Enzyme Concentration

The initial enzyme concentration is also an important factor that not only influences enzyme immobilization, but also affects the retention activity of IE. After the carrier is saturated with loaded enzyme, the immobilization efficiency will decrease with increasing initial enzyme concentration. In the present study, the fixation level was kept almost constant within the whole range of initial enzyme concentration (Fig. 3a), and the amount of the enzyme fixed on Lew continually increased at higher initial enzyme concentration (Fig. 3a). This indicates that the capacity of Lew to bind the enzyme is high, i.e., Lew has the potential to immobilize more enzyme.

In order to see the effect of enzyme loading on the activity of IE, the catalytic activity of the immobilized PLC was measured, and the result is shown in Fig. 3b. The specific activity of IE was essentially unchanged when the amount of enzyme in the carrier was low, but increased linearly when the mass of enzyme in the carrier was above 11 $\mu\text{g/mg}$. Generally, when the amount of enzyme immobilized on the carrier is increased, the specific activity of the enzyme will decrease owing to the increased steric restrictions of the enzyme and the mass-transfer limitation of substrates and products [12]. Therefore, the current result is abnormal, and only one similar case has been found, in the immobilization of lipase [14]. This phenomenon can be explained in two ways:

1. Certain “dead pores” exist in the carrier, and the enzyme can be inactivated by contacting with these dead pores. Since “nondead pores” exist in the

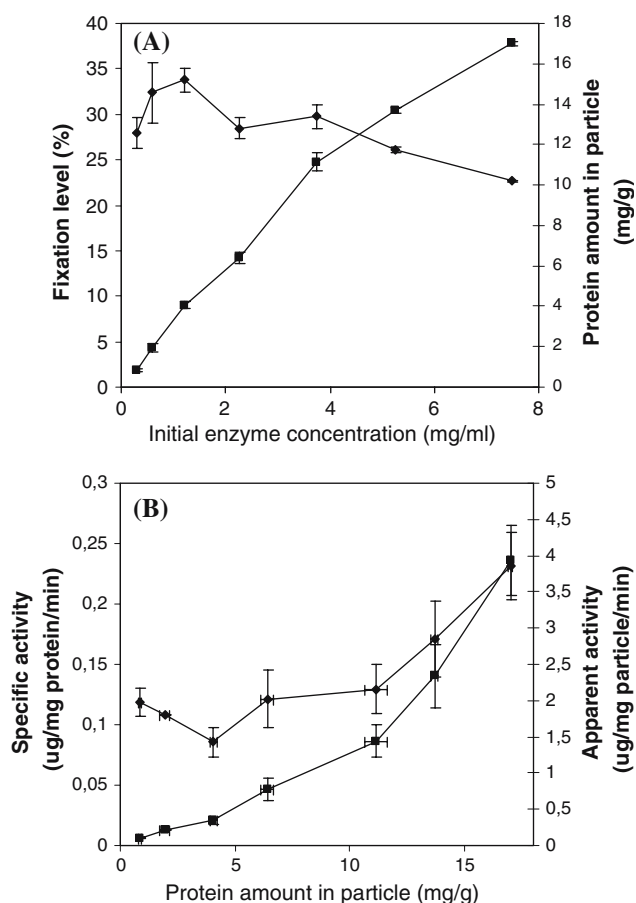


Fig. 3 The influence of initial enzyme concentration on the immobilization process. The immobilization procedure and the activity test of IE are described in the “Materials and Methods.” **a** Diamonds the fixation level and squares the amount of protein immobilized on Lew for different initial enzyme concentrations. **b** Diamonds specific activity and squares apparent activity on different protein loading

carrier too, enzyme activity can still be detected at low enzyme loading. When the amount of the enzyme in Lew was increased, these dead pores were saturated by the enzyme. After the saturation point, the new adsorbed enzyme exhibited the intrinsic activity. Therefore, the whole specific activity of IE was increased when more enzymes were immobilized on the particles.

- Since the amount of the carrier was unchanged, the accessible surface area of the carrier was fixed. The increase of the amount of the enzyme in Lew decreased the accessible surface area per enzyme molecule, leading to less contact of individual enzyme molecules with Lew. As a result, the extent of inactivation caused by this contact was reduced, and the specific activity of IE was increased with more enzymes adsorbed in Lew.

By controlling the initial enzyme concentration, we can obtain the same amount of enzyme on the carriers as when pretreating Lew with ethanol; however, both the specific activity and the apparent activity of IE were high compared with those for the ethanol-treated Lew (Fig. 3b). This shows that the inactivation of IE, induced by the ethanol treatment on Lew, is not due to the increased amount of the enzyme on the carriers. Therefore, the first explanation for the inactivation of IE caused by ethanol treatment can be rejected, and the mass-transfer limitations as well as the steric restrictions of the enzyme do not seem to play a vital role in catalytic reaction from the current enzyme loading. The catalytic activity of IE depends more on the accessible surface area of the carrier, which mainly governs how the enzyme is distributed in the pores of the carrier [13]; hence, the geometric characteristic of the carrier is an important factor in determining the catalytic activity of IE.

Optimization of Immobilized Enzyme Reaction Using Response Surface Methodology

So far, the immobilization process was optimized with the considerations of the fixation level and the catalytic activity of IE. By evaluating the influence of carrier type, ethanol treatment, and initial enzyme concentration, we selected the following immobilization conditions for further characterization: Lew as the carrier, no ethanol treatment and initial enzyme concentration of 0.75 mg/ml.

Temperature and pH optima are two basic properties of an enzyme. The optimization of temperature and pH for soluble enzyme has already been addressed [5]. The immobilization of an enzyme often changes the temperature and pH profiles. In addition, the water content in the reaction medium is also an important factor whose optimum is often subjected to the change induced by immobilization. These issues can be better solved through an optimization operation assisted by RSM. RSM is an effective statistical technique for the optimization of complicated systems, and enables the evaluation of effects from multiple parameters, alone or in combination, on response variables. The main advantage of RSM is the reduced number of experiments needed to provide sufficient information for statistically acceptable results [15]. The three parameters and their ranges in Table 2 were decided on the basis of previous studies. The responses were first fitted to the parameters. The best-fitting quadratic model was determined by regression and backward elimination. The model coefficients and the *P* values for the regression variables are given in Table 4. All *P* values

Table 4 Regression coefficients describing the influence of different parameters on initial reaction rate ($\mu\text{g}/\text{min}$)

Variables	Coefficient	Standard error	P value	Conf ^a
Constant	29.16	1.57	1.17×10^{-9}	3.45
Temperature	6.83	1.17	1.12×10^{-4}	2.57
H ₂ O volume	−6.87	1.17	1.04×10^{-4}	2.57
Temperature \times temperature	−3.91	1.17	6.65×10^{-3}	2.58
Temperature \times H ₂ O volume	−8.13	1.27	5.24×10^{-5}	2.81

^a The 95% confidence interval (\pm) on the coefficient value

of the coefficients were below 0.01 after the model had been refined. The coefficient of determination (R^2), which is a measure of fit, was 0.92. In the normal probability plot of residuals, there was a good linear relationship for all the points within a narrow range of standardized residues (−1.4 to 1.7) (Fig. 4). This shows that the model generally represented the actual relationships between the response and the reaction parameters within the ranges selected.

Figure 5a is a summary of the effects on the reaction based on the model defined. Since the pH and its interactions with the other factors had little effect on the catalytic reaction of IE, they were eliminated from the model. The effect of pH on the catalytic reaction is discussed in the next section. Temperature and water volume had a significant influence on the SM hydrolysis. The interaction between these two factors also had a big negative effect on the reaction. With the aim of providing a clear evaluation of the two significant parameters, the main effects of each parameter are given in Fig. 5b and c. Theoretically, the higher the temperature, the greater the reaction rate that can be reached when other aspects, such as thermal inactivation

of enzyme and thermal degradation of substrates and products, can be ignored. From the result, the optimal temperature was around 46 °C, and the initial reaction rate was not increased beyond 46 °C (Fig. 5b). When the optimal temperature of the IE is compared with that of the soluble enzyme, which has the highest SM hydrolysis around 37 °C [5], we can conclude that the immobilization process increases the optimal temperature of the reaction. Because immobilization provides a more rigid external backbone for enzyme molecules, the proper and catalytically active structure of the enzyme becomes more stable at higher temperatures [6]. Accordingly, the inactivation of PLC at higher temperature becomes less significant, and the optimal temperature will increase.

Water not only plays the role of a nucleophile in the reaction, but also contributes to the active state of the enzyme [16]; therefore, the amount of water has a large impact on SM hydrolysis catalyzed by soluble PLC [5]. In present study of IE, the initial reaction rate and the water volume exhibited a negative linear relationship (Fig. 5c), when the other factors in the design were set to their average. An excess amount of water possibly assists the thermal inactivation of IE, leading to the decrease in the catalytic activity at high temperatures. In order to examine this assumption, the relationship and the interaction between temperature and water volume are illustrated by the contour plot (Fig. 5d) when the other parameter in the design was set to its average. At lower temperatures, the water volume had little effect on the reaction rate, while at higher temperatures, increasing water volume was strongly inhibitory. This indicates that the negative effect of water volume was temperature-dependent. Furthermore, when the water volume was low, increasing temperature enhanced SM hydrolysis, and thermal inactivation was reduced. This was reversed when the water volume was high. In conclusion, it is verified that a high water volume makes the IE reaction more vulnerable to thermal deactivation, which is responsible for the negative effect of the water volume on SM hydrolysis.

Through iterative calculation to determine the maximum initial reaction rate, the results recommend the general optimal conditions as water volume 70 μl and temperature 46 °C. Under these conditions, the initial reaction rate should be 52.2 $\mu\text{g}/\text{min}$ according to the prediction of the model.

Temperature and pH

The hydrolysis reaction of IE was optimized using RSM, including three important factors, temperature,

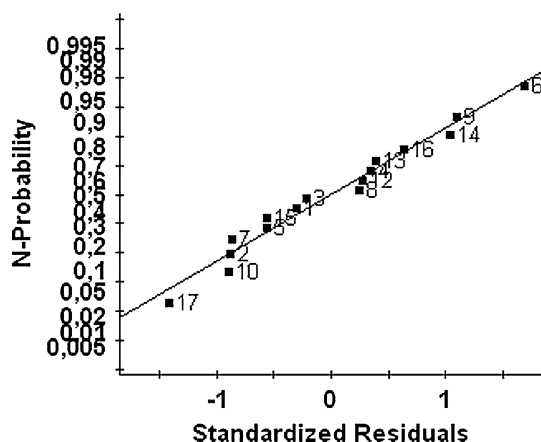
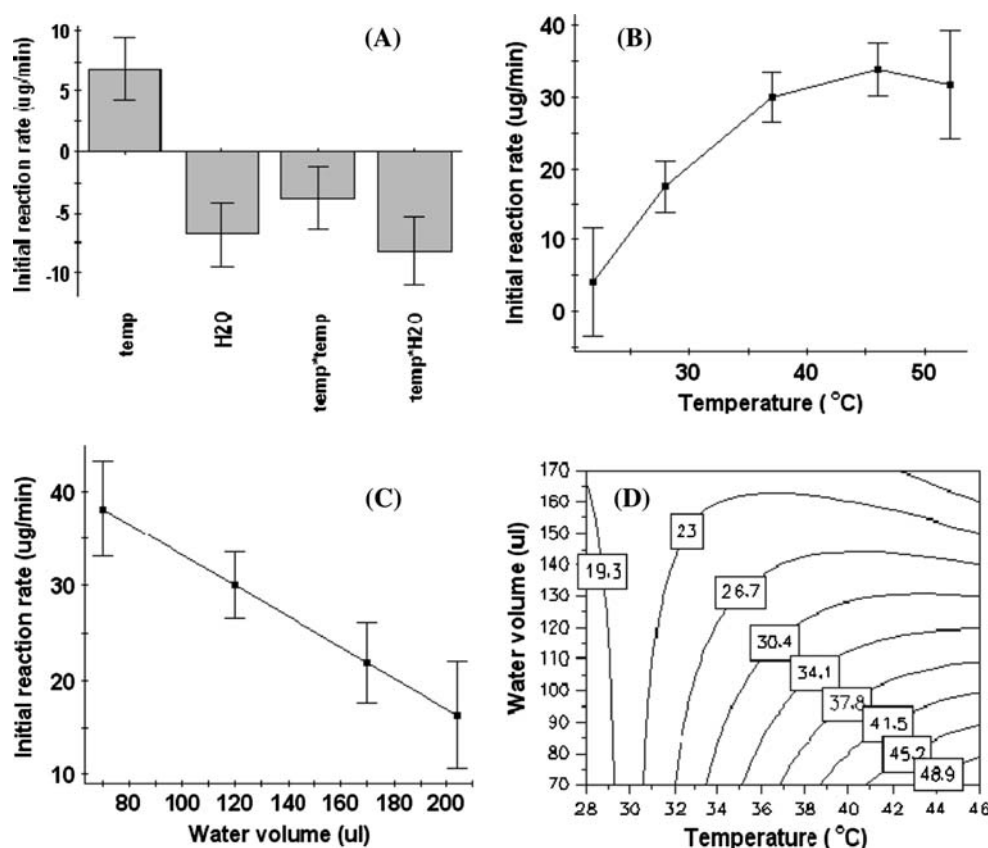


Fig. 4 The normal probability plot of residuals. The numbers represent the number of the experiment

Fig. 5 The results from the optimization study for IE reaction using response surface methodology. Three factors, temperature, water volume, and pH, were selected for optimization. The only response was the initial reaction rate. **a** Summary of the effects on SM hydrolysis. Owing to their having little effect, the pH and its interactions with other factors were eliminated from the model. **b, c**, Main effect of temperature and water volume, respectively. The plot of the main effect displays the predicted change in the response when the factor varies from its low to its high level, all other factors in the design being set to their average. **d** The contour plot between temperature and water volume. The numbers indicate the initial reaction rate ($\mu\text{g}/\text{min}$)



pH, and water volume, in the analysis. Optimal reaction conditions were generated through the model. However, the model evaluates the effects on response variables through multiple parameters, and evaluation of the effects of each factor will influence the evaluation of the other factors, even though in a good model the predicted value is almost same as the observed value. To get the exact profile of the effect, the factor has to be optimized, with other parameters fixed to optimal values; therefore, the two basic factors, pH and temperature, were further evaluated individually.

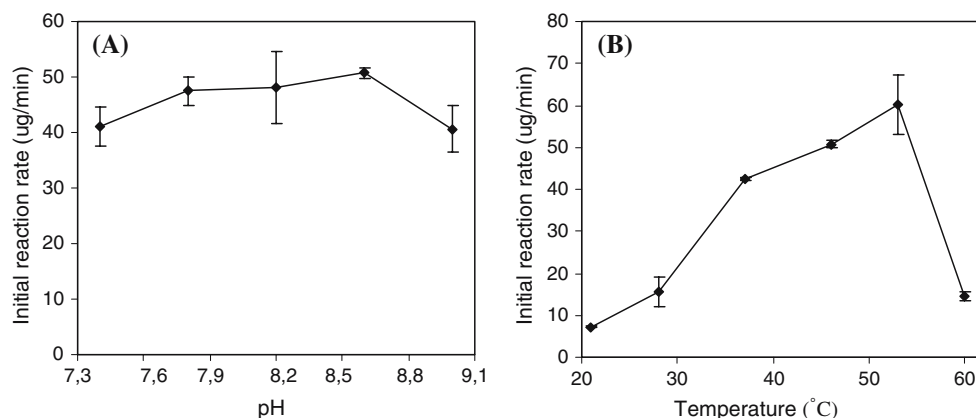
In the present study, the effect of pH was tested in a range from 7.4 to 9. The initial reaction rate varied insignificantly within the selected pH range (Fig. 6a); therefore, the enzymatic reaction of IE has little dependence on pH. The negligible effect of pH on SM hydrolysis was concurrent with the result from the model described above, and this concurrency indicates the model is valid. The result differed from what was observed in the soluble enzyme, where the reaction is sensitive to pH shifts [5]. Similar pH tolerance, induced by enzyme immobilization, has been reported in a study of phospholipase D immobilization [11]. The possible explanation is that adsorption of the enzyme in the carrier can shield the charge state of the substrate or the enzyme, so pH changes in the medium are

less effective in altering protonation [11]. Because of this pH tolerance, we can conduct the reaction within a broad pH range, and the choice of pH can be subjected to the specific preference. Therefore, the small pH effect is advantageous for utilization of PLC. The selected temperature range for evaluation was from 21 to 60 $^{\circ}\text{C}$. The optimal temperature was between 46 and 53 $^{\circ}\text{C}$, and the initial reaction rate was dramatically decreased beyond 53 $^{\circ}\text{C}$ (Fig. 6b). The decrease in the reaction rate at higher temperature is possibly due to the thermal inactivation of the enzyme.

Operational Stability Test

In the present optimization study using IE for the reaction, the optimal conditions included a temperature of 46 $^{\circ}\text{C}$ and a water volume of 70 μl ; therefore, these conditions were applied in the following stability study. The operational stabilities were compared among reactions with the soluble enzyme and the IE at 46 and 37 $^{\circ}\text{C}$. In the soluble enzyme catalyzed reaction, only the water phase of the reaction mixture was recycled, meaning that some water might be dissolved in the organic solvent and therefore lost. After the fourth batch, the water phase disappeared in the reaction mixture, and no ceramide was detected

Fig. 6 Influence of pH (a) and temperature (b) on SM hydrolysis using immobilized phospholipase C as a catalyst



(Fig. 7a). The decrease in the volume of the enzyme solution due to solubility of water in the organic solvent contributes the activity loss of the enzyme in each cycle. Therefore, PLC in soluble form is difficult to reuse under the present conditions. For the operational stability test of IE, the reusability of the enzyme was reaction temperature dependent (Fig. 7a). After recycling, IE remained more active at 37 °C than at 46 °C, verifying that the thermal inactivation of IE plays an important role in the reuse of the enzyme. Since the initial reaction rate was higher at 46 °C from the optimization study, a compromise has to be made between the reaction rate and the stability of IE. With a reaction temperature of 37 °C, the activity of IE was even enhanced by the recycling; thus, the activity was higher in the second and third batches than in the initial batch. This activation is probably due to the conformation change of the enzyme in the enzyme-conditioning step. In the eighth batch, IE retained around 70% of the initial activity; therefore, the reusability of the enzyme is greatly improved by immobilization.

Storage Stability Test

The storage stability test was conducted under three different storage conditions: PLC dissolved in buffer solution at room temperature, IE at room temperature, and IE at 40 °C. The activities of the enzyme at different storage times were irregular when both PLC was dissolved in the solution and IE was stored at room temperature (Fig. 7b). The catalytic activities increased for some days at the beginning, and this phenomenon probably has the same principle as the IE activation induced by recycling in the operational stability test study. When IE was stored at 40 °C, the activity of the enzyme decreased with the storage time. The plot of activity against storage time followed the equation of the first-order reaction of thermal inactivation:

$$[E_t] = [E_0]e^{-k_D t}, \quad (2)$$

where $[E_t]$ is the enzyme activity at time t , $[E_0]$ the original enzyme activity without heating, and k_D the

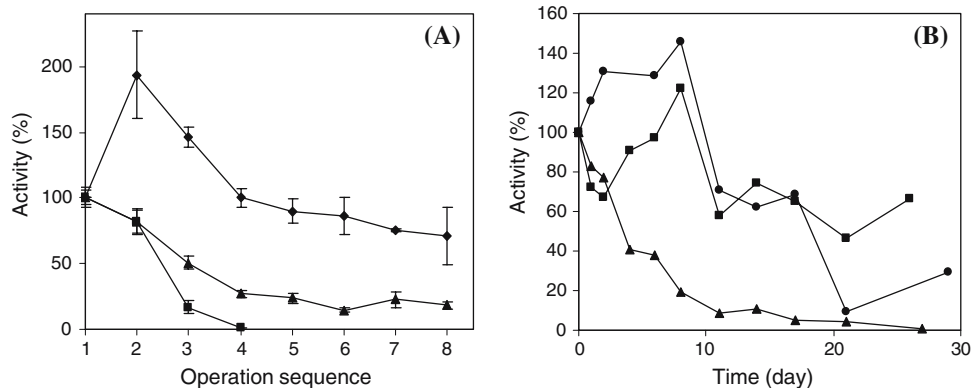


Fig. 7 Stability test for immobilized phospholipase C. The y-axis is the percentage of the original catalytic activity that was calculated from the initial reaction rate. **a** Operational stability test. *Squares* soluble enzyme reaction, *Diamonds* IE reaction at

37 °C, and *triangles* IE reaction at 46 °C. **b** Storage stability test. *Squares* soluble phospholipase C stored in buffer solution at room temperature, *circles* IE stored at room temperature, and *triangles* IE stored at 40 °C

first-order inactivation rate constant. When the data were fitted to the equation with the intercept set to 100%, k_D was calculated to be 0.17 per day, and the coefficient of determination (R^2) was 0.97. Through this equation, the characteristic half time for thermal inactivation was $\ln(2/k_D) = 4.1$ days. The rate constant, k_D , which is characteristic for the reaction conditions at the given temperature, depends on many factors, such as temperature, pH, substrates, and enzymes. The effect of temperature on k_D can be examined by tests with different temperatures, with other parameters fixed. However, since the results from the storage stability tests at room temperature were irregular in the present study, it is difficult to obtain the profile of the influence of temperature on k_D .

In summary, PLC immobilized on Lew had the highest catalytic activity towards SM hydrolysis. The geometric characteristics of the carrier are a crucial factor that influences the observed catalytic activity of IE. From the results of the optimization study and the stability test of IE reaction, immobilization of the enzyme improved the performance of the SM hydrolysis reaction. Application of the immobilized PLC to produce ceramide from SM modification seems to be attractive and will be the objective of future studies.

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PAPER III

Long Zhang, Lars I. Hellgren and Xuebing Xu (2008) Kinetic study of sphingomyelin hydrolysis for ceramide production, *Journal of Molecular Catalysis B: Enzymatic*, 51: 93-99



Kinetic study of sphingomyelin hydrolysis for ceramide production

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Abstract

Kinetic study of sphingomyelin hydrolysis catalyzed by *Clostridium perfringens* phospholipase C was, at the first time, conducted for ceramide production. Ceramide has the major role in maintaining the water-retaining properties of the epidermis. Hence, it is of great commercial potential in cosmetic and pharmaceutical industries such as in hair and skin care products. The enzymatic hydrolysis of sphingomyelin has been proved to be a feasible method to produce ceramide. The kinetic performance of sphingomyelin hydrolysis in the optimal two-phase (water:organic solvent) reaction system was investigated to elucidate the possible reaction mechanism and also to further improve the hydrolysis performance. Enzyme in solution had less thermal stability than the enzyme powder and the immobilized enzyme. The thermal inactivation of phospholipase C in all the three forms did not follow the first order reaction at 65 °C. The reactions for both the soluble and immobilized enzymes followed Michaelis–Menten kinetics. K_m 's for the soluble and immobilized enzymes were 1.07 ± 0.32 and 1.26 ± 0.19 mM, respectively. The value of V_{max} was markedly decreased by the immobilization without much change in K_m , as if the immobilization functioned as the non-competitive inhibition. Ceramide as product activated the hydrolysis reaction, however, and its addition mainly caused the increase in the affinity of the enzyme–substrate complex. © 2007 Elsevier B.V. All rights reserved.

Keywords: Ceramide; Phospholipase C; Sphingomyelin; Kinetic; Thermal stability

1. Introduction

Ceramide (Fig. 1A) is a key intermediate in the biosynthesis of all complex sphingolipids. Due to its major role in maintaining the water-retaining properties of the epidermis [1–3], ceramide is of great commercial potential in cosmetics and pharmaceuticals such as hair and skin care products. Many ceramide-containing products have already been introduced in the cosmetic market, and the effect of its application is excellent. However, chemical synthesis of ceramide is a costly and time-consuming process for industrial applications. Therefore, developments of alternative cost-efficient and high-yield production methods are of substantial interest. Sphingomyelin (SM) is a ubiquitous component of animal cell membranes and it is one of the major phospho-

lipids in bovine milk. In SM, the ceramide part of the molecule is bound through a phosphodiester bridge to a choline moiety (Fig. 1B). Systematical investigation and optimization have been conducted for the enzymatic production of ceramide from SM hydrolysis [4]. Phospholipase C (PLC, EC 3.1.4.3) from *Clostridium perfringens* shows high activity towards the hydrolysis reaction. The hydrolysis is more efficient in the two-phase (water:organic solvent) system than in the one-phase (water-saturated organic solvent) system [4].

For continuous development of ceramide production, it is necessary to consider the mechanism of the catalytic reactions. Kinetic studies are powerful tools to elucidate the possible reaction mechanisms and improve the hydrolysis in order to obtain the highest reaction rate. Kinetic study for the hydrolysis of SM in monolayer has been focused when sphingomyelinase (EC 3.1.4.12) was used as the catalyst [5]. Kinetic models have been established to illuminate the possible mechanisms for the interfacial activation of the sphingomyelinase. However, due to the need of larger operation area, the monolayer reaction is not quite feasible for ceramide production in industrial applications. Hence, kinetic study is required for the reaction system specific to the ceramide production. In addition, since the hydrolysis catalyzed by the PLC or its immobilized form was better at

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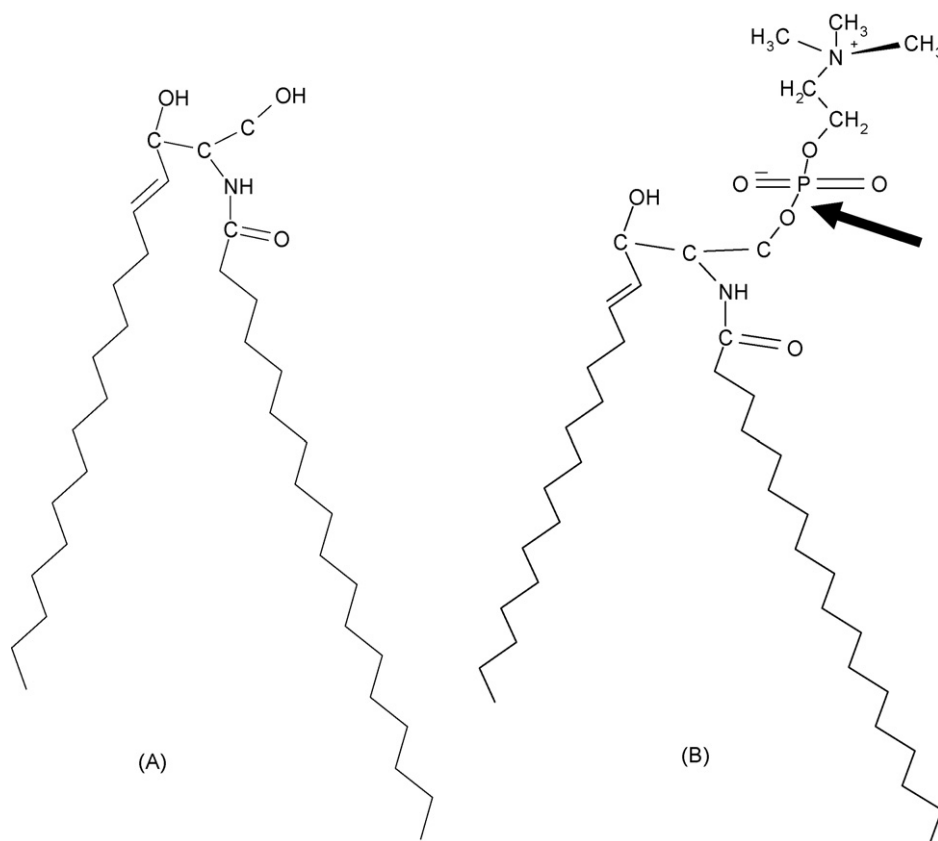


Fig. 1. The structures of ceramide (A) and sphingomyelin (SM) (B). The arrow indicates the bond to be cleaved in SM for producing ceramide.

high temperatures [4,6], thermal stability of the enzymes is also critical.

Present study aims to investigate the kinetic behaviour of SM hydrolysis catalyzed by PLC from *C. perfringens*, with the two-phase reaction system as the model system. Thermal stability was examined for the enzyme powder, the enzyme in solution, and the immobilized enzyme (IE). Enzyme and substrate concentration effects on the hydrolysis were also evaluated. Finally, product activation was found in the current reaction system instead of inhibition.

2. Experiment

2.1. Materials

Bovine brain SM (approx. 99%), standard ceramide ($\geq 99\%$) from bovine brain and *C. perfringens* phospholipase C (Type I) were purchased from Sigma–Aldrich Denmark A/S (Copenhagen, Denmark). The molecular weight of bovine brain SM is 776 [7]. The carrier, Lewatit VP OC 1600 (Divinyl benzene crosslinked polymer), was from Bayer AG (Leverkusen, Germany). Standard or other chemicals were of analytical and chromatographic purity.

2.2. Immobilization process

The immobilization process of PLC has been described previously [6] with minor change. Carrier (350 mg) was

conditioned in 6 ml 96% ethanol for 20 min, followed by washing twice with 6 ml distilled water and drying in fume hood overnight. One-hundred milligrams of carrier was suspended in 1 ml enzyme solution (0.75 mg protein/ml 0.01 M Tris(hydroxymethyl)amino-methane (Tris–HCl buffer, pH 8.6)). The mixture was shaken in a rotary shaker (Bie & Berntsen, Rødovre, Denmark) at 600 rpm for 17 h. Lower clear solution (0.3 ml) was removed for the analysis of protein concentration according to the Lowry method [8] using bovine serum albumin as the standard. The solids in the mixture was obtained through vacuum filtration and washed three times with 3 ml buffer solution. IE was dried through 5 min vacuum followed by 3 h air conditioning in the fume hood.

2.3. Enzymatic activity test

Reaction conditions for the soluble and immobilized enzymes were essentially the same as described previously [4,6]. According to substrate concentration planned (e.g. 0.52 mM), certain amount of SM was mixed with ethylacetate:hexane (50:50), and the mixture was placed in an ultrasonic machine (Bie & Berntsen, Rødovre, Denmark) for 5 min at 37 °C. For the soluble enzyme, 200 μ l substrate solution was introduced in the reactor, a small glass tube (diameter, 0.9 cm; height, 7.4 cm). The reaction was started by adding 12 μ l 0.026 mg/ml enzyme solution (in 0.01 M Tris–HCl buffer (pH 8.6) containing 25% ethanol). The reactor was sealed by a cap, and kept at 37 °C with shaking at 200 rpm. For the IE, 5 mg IE (containing 12.5 μ g enzyme) and

7 μ l Tris–HCl buffer with 25% ethanol were mixed. Substrate solution (200 μ l) was added to start the reaction, which was kept at 46 °C. Other procedures were the same as described above. After reaction, shaking was stopped, and 80 μ l reaction mixture from the upper phase (organic solvent phase) was withdrawn and stored at –20 °C until analysis. Reaction time was controlled to make sure that the detected reaction rate was initial reaction rates. All values given were averages from duplicate experiments, and standard deviation was used as error bar in the figures.

2.4. Thermal stability test

The enzyme powder, the enzyme solution (0.104 mg/ml in buffer containing 25% ethanol) and the IE were put in the individual Eppendorf tubes and heated for 10 min at different temperatures. The enzyme solution and the IE were directly used for the activity test. The heated enzyme powder was dissolved into enzyme solution (0.104 mg/ml) before the test. The concentration of the substrate solution used was 0.52 mM. Other procedures were the same as described above.

2.5. Kinetic study

The data were fitted to Michaelis–Menten kinetic model as below:

$$V = \frac{V_{\max}[S]}{[S] + K_m} \quad (1)$$

where V is the initial reaction rate, $[S]$ the initial substrate concentration, V_{\max} the maximum initial reaction rate and K_m the Michaelis constant. The software Prism 5 (Graphpad Software, Inc., San Diego, USA) was used to facilitate the fitting of the data and the calculation of K_m and V_{\max} .

2.6. Analysis method

Ceramide concentrations were quantified using high performance thin-layer chromatography (HPTLC) and in situ densitometry, essentially as earlier described [9]. The HPTLC-plate (Silica gel 60, E. Merck, Darmstadt, Germany) was pre-washed through development in chloroform:methanol (2:1 by volume) in a horizontal developing chamber (Camag, Muttenz, Switzerland), and the plate was activated at 120 °C for 30 min. Standards and samples were applied using DESAGA AS30 HPTLC Applicator (Sarstedt Ag & Co., Nümbrecht, Germany). Following equilibration and development with heptane:isopropanol:acetic acid (85:15:1) in the chamber, the plate was dried and sprayed with charring reagent (10% CuSO_4 in 8% H_3PO_4) until soaked. Lipids were visualized by heating the plate at 160 °C for 6 min. The intensity of the spots was determined using DESAGA CD60 HPTLC Densitometer (Sarstedt Ag & Co., Nümbrecht, Germany) at 390 nm in the absorbance/reflection mode. The lipid mass of each component was calculated with the software DESAGA ProQuant (1.03.200, Sarstedt Ag & Co., Nümbrecht, Germany) using a standard calibration curve run on the same plate. Initial reaction rate was the ceramide concentration divided by the reaction time.

3. Results and discussion

3.1. Thermal stability

Thermal stability was studied to investigate temperature influence on the catalytic activity of the enzyme. After heating the enzyme in three different forms, i.e. enzyme powder, enzyme in solution, and the IE, their catalytic activities were measured individually in standard conditions. The effect of heating temperature on the catalytic activity was shown in Fig. 2. Enzyme in solution was most vulnerable by the increase of heating temperature. The heat transfer efficiency in solution is much better than in enzyme powder and IE. This could enhance the thermal inactivation of enzyme in solution. In addition, as happened with most proteins, thermal unfolding of the enzyme in solution lead to the aggregation and the loss of the intrinsic structure. Hence, the enzyme in solution became inactivated at higher temperatures.

The effect of heating period on the catalytic activity was evaluated at heating temperature 65 °C. The catalytic activity of the enzymes in all three forms decreased with the prolongation of the heating time (Fig. 3A). As the reason discussed above, the enzyme in solution was denatured very quickly by heating. The enzyme powder was less affected by heating time than the IE. This implied that the enzyme powder had high thermal stability comparing with the IE. Due to a more rigid external backbone for enzyme molecules, the IE should be stable at higher temperatures [10]. In this study, the protein purity in the enzyme powder from the company was 0.52 mg protein/mg powder, as measured by the Lowry method [8]. Therefore, protein stabilizing substances, e.g. ions or sugars, might exist in the enzyme powder. These substances can protect the enzyme from the thermal denaturation. However, they might be lost during the immobilization process of the enzyme. As a result, the IE had less thermal stability than the enzyme powder.

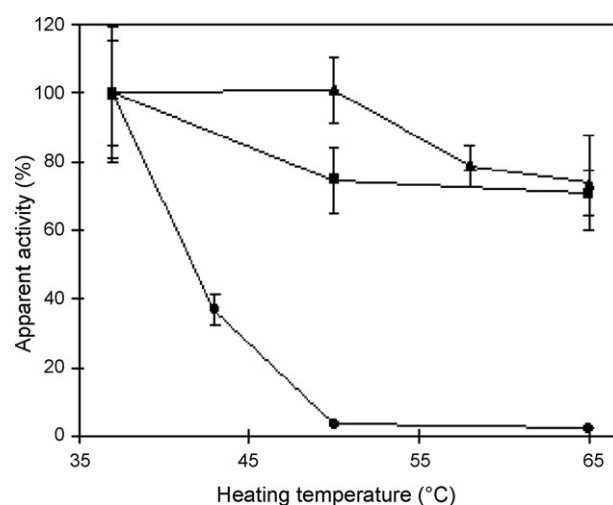


Fig. 2. The effect of heating temperature on the catalytic activity of enzymes. After heating the enzyme powder (■), the enzyme in solution (●) and the immobilized enzyme (▲) for 10 min at different temperatures, their catalytic activities were measured as described in Section 2. Apparent activity was expressed as a percentage of the initial reaction rate of enzymes heated at 37 °C.

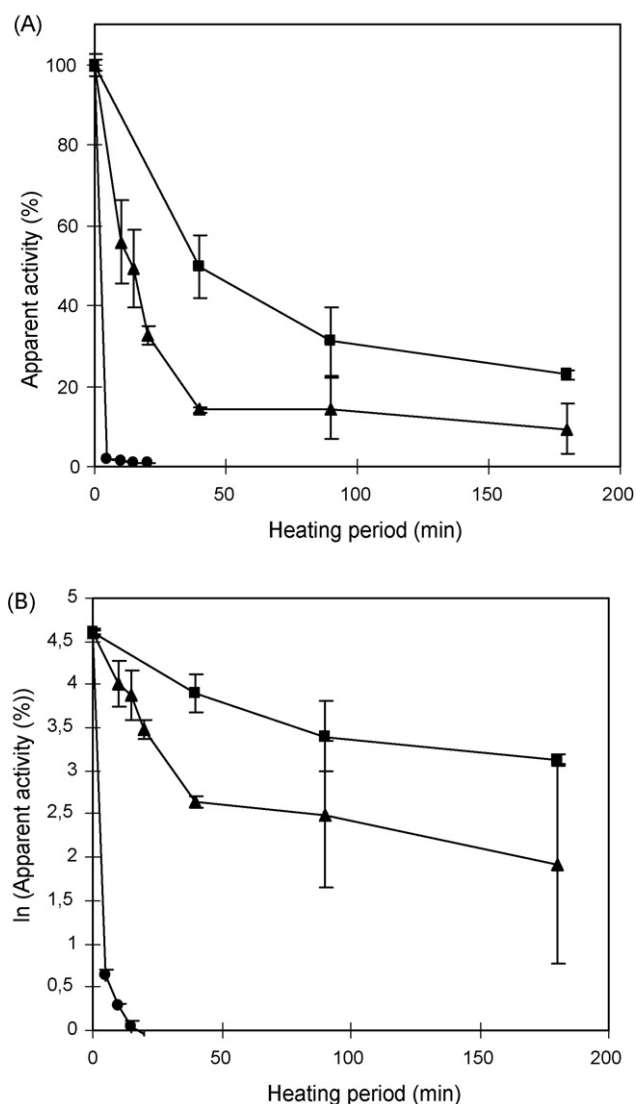


Fig. 3. The effect of heating time on the catalytic activity of enzymes at heating temperature 65 °C. The legend was the same as Fig. 2. Apparent activity was expressed as a percentage of the initial reaction rate of enzymes without heating. (A) Normal plot and (B) the logarithm plot.

Usually in thermal inactivation process, the activity plot against heating time followed the equation of the first order reaction:

$$[E_t] = [E_0]e^{-k_D t} \quad (2)$$

where $[E_t]$ is the enzyme activity at heating time t , $[E_0]$ the original enzyme activity without heating and k_D is the first order inactivation rate constant. Below is the logarithm form of Eq. (2), which can be used to calculate k_D .

$$\ln[E_t] = \ln[E_0] - k_D t \quad (3)$$

Thus, if the plot of $\ln[E_t]$ against t is linear, the inactivation follows the first order reaction, and k_D can be determined from the slope. From Fig. 3B, all plots were not linear, indicating that the thermal inactivation of enzymes in all three forms did not follow the first order reaction at 65 °C. In the previous study

[6], the thermal inactivation of immobilized PLC is irregular at the room temperature and follows the first order reaction well at 40 °C. Therefore, heating temperature has a crucial effect on the performance of the thermal inactivation of the immobilized PLC. When we evaluated the outcome in different heating periods, the plot of the immobilized PLC was linear at heating time from 0 to 40 min. After 40 min, the line turned to be flat until 180 min. Hence, its property of the thermal inactivation also depended on heating period. During heating, the microenvironment of the enzyme molecule in the IE could change, or this change might accumulate. Accordingly, the decrease of the catalytic activity of the enzyme became slow in certain heating time. Consequently, k_D value was higher in early stage than in late stage for the IE.

3.2. Enzyme concentration effect

When substrate concentration is much higher than enzyme concentration and other factors such as mass transfer limitation can be ignored, the increase of enzyme amount will make the reaction faster. The effect of enzyme concentration for the soluble enzyme was evaluated with the fixed SM concentration (0.52 mM). The initial reaction rate was proportional to enzyme concentration in the test range (Fig. 4). Therefore, the current reaction system could be a new method for the catalytic activity assay of PLC. The general activity assays for PLC use aqueous system. Only one assay of *B. cereus* PLC works in the two-phase (chloroform:buffer) system, where phosphatidylcholine is the substrate [11]. In that assay, two-phase system provides high efficiency for the hydrolysis reaction. However, when chloroform was used with buffer in the present two-phase system, *C. perfringens* PLC had little catalytic activity. Therefore, we concluded to use the organic solvent mixture, ethylacetate:hexane (50:50), for the two-phase system.

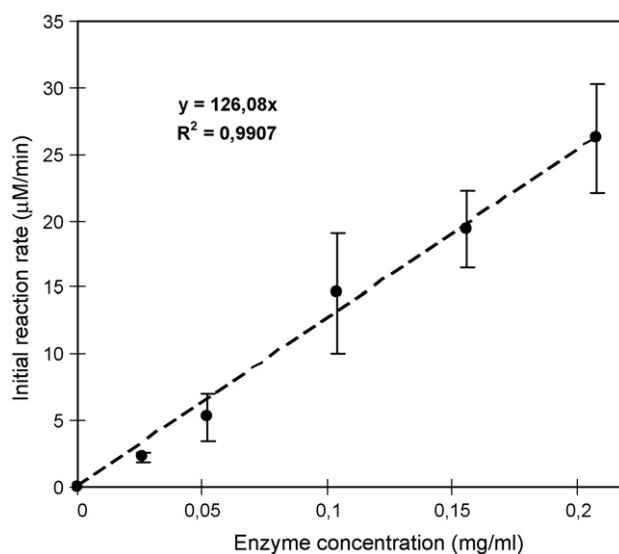


Fig. 4. Enzyme concentration effect on the hydrolysis reaction. The soluble enzyme was used to catalyze the reaction at a fixed sphingomyelin concentration (0.52 mM). The X-axis was the enzyme concentration in the water phase.

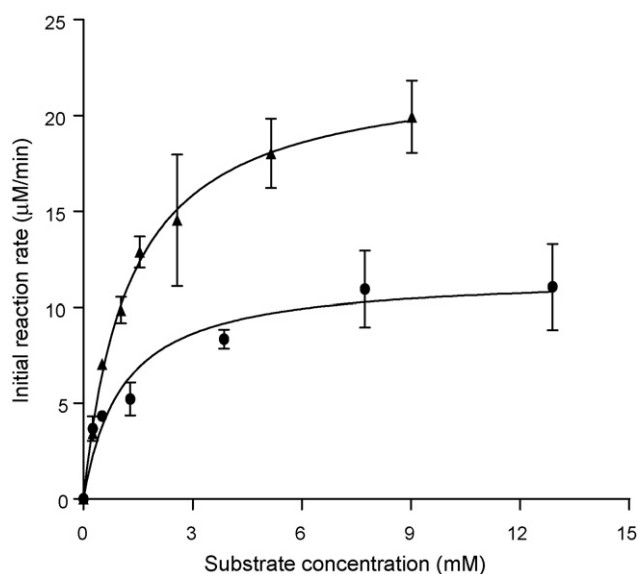


Fig. 5. Substrate concentration effect on the hydrolysis reaction. The plots were drawn using the Michaelis–Menten kinetic model assisting with the software Prism 5. (●) Soluble enzyme and (▲) immobilized enzyme. The amount of enzyme used was 0.31 μg (for the soluble enzyme) and 12.5 μg (for the immobilized enzyme).

3.3. Substrate concentration effect

Since water molecule is abundant in the hydrolysis reaction, its concentration has no significant effect on the kinetic behaviour of the reaction. As a result, we can focus on the effect of SM concentration, and the hydrolysis can be simplified as one-substrate reaction for kinetic study. Consequently, Michaelis–Menten kinetics can possibly be applied in the enzymatic reaction. SM concentration effect on the hydrolysis reaction was examined with the fixed enzyme amount. The reactions in both the soluble and immobilized enzymes followed Michaelis–Menten kinetics (Fig. 5). Kinetic parameters, K_m and V_{\max} , were summarized in Table 1 for both enzymes. The amount of the enzyme used was different between the soluble enzyme (0.31 μg) and the IE (12.5 μg). Therefore, in table we used V_{\max} divided by the amount of the enzyme in order for a clear comparison.

When an analogue of lysophosphatidylcholine was the substrate, the hydrolysis reaction catalyzed by *C. perfringens* PLC also followed Michaelis–Menten kinetics in the micellar reaction system [12]. K_m was 36 μM from the study and was different from the present study ($1.07 \pm 0.32 \text{ mM}$ for the soluble enzyme). The Michaelis constant, K_m , is normally interpreted as the dissociation constant of the enzyme–substrate complex, and a low K_m value represents a high affinity of the enzyme to the substrate. Therefore, the big difference in K_m indicated that the affinity of

the enzyme–substrate complex was lower in the present study. This is possibly caused by the difference in the substrate between the two studies. The other possible reason is the discrepancy in the reaction system. Enzyme molecule in buffer can directly bind the substrate micelle in the micellar system. In contrast, enzyme molecule had to stay in the interface between two phases to catch the substrate in the two-phase system used for the present study. However, two-phase system is superior to micellar system for phosphatidylcholine hydrolysis catalyzed by *B. cereus* PLC [11]. Hence, it has no conclusion on which reaction system is the best for the catalytic activity of the enzyme in current applications. In plant scale productions, the separation of ceramide from the micelle and the requirement of high-speed stirring to prevent aggregate formation make the practice more difficult in micellar systems. Thus, two-phase system would be preferable for the ceramide production in practical operations.

When kinetic parameters were compared between the IE and the soluble enzyme, only V_{\max} had significant difference (Table 1). Small variation in K_m indicated that the affinity of the enzyme to the substrate was little affected by the immobilization. The reduction of V_{\max} for the IE without much change in K_m implied that the immobilization probably functioned as non-competitive inhibition. A non-competitive inhibitor binds the enzyme at a site distinct from the substrate, and the binding is independent on the enzyme–substrate binding [13]. Therefore, it could be deduced that the carrier contacted the enzyme molecule not mainly through the active site of the enzyme. The decrease of the reaction rate in the IE might be caused by the reduced moving capability of the enzyme due to the immobilization. However, the reduction of V_{\max} for the IE without much change in K_m can also be from the other reason. After immobilization, some enzymes might have the same values of K_m and V_{\max} as the soluble enzyme, while other enzymes could lose most of the catalysis capability by immobilization. Therefore, the apparent K_m only showed the K_m of the former enzymes, where the value would be similar to the soluble enzyme. Since the total catalytic activity was reduced by immobilization, V_{\max} was significantly decreased.

According to data in Table 1, the catalytic efficiency, V_{\max}/K_m , was decreased to 4.1% by the immobilization of the enzyme. This seems to be unfavourable for the practical use of the IE. However, the soluble enzyme is difficult to be reused, and the IE retains around 70% of the initial activity after seven cycles [6]. Other benefits of the immobilization is that the reaction catalyzed by the IE has little dependence on pH and has a higher temperature optimum than the reaction catalyzed by the soluble enzyme [6]. The price of PLC is high. Therefore, using the immobilization method to improve the reusability of the enzyme would considerably reduce enzyme cost in industrial applications.

3.4. Product effect

Product effect on the hydrolysis catalyzed by the soluble enzyme was evaluated through the addition of ceramide (0.33 mM) before the reaction in different SM concentrations. Obviously but surprisingly, ceramide activated the hydrolysis

Table 1
Comparison of kinetic parameters for the soluble and immobilized enzymes

Enzyme type	K_m (mM)	V_{\max} ($\mu\text{M}/(\mu\text{g enzyme min})$)
Soluble enzyme	1.07 ± 0.32	37.68 ± 2.91
Immobilized enzyme	1.26 ± 0.19	1.80 ± 0.09

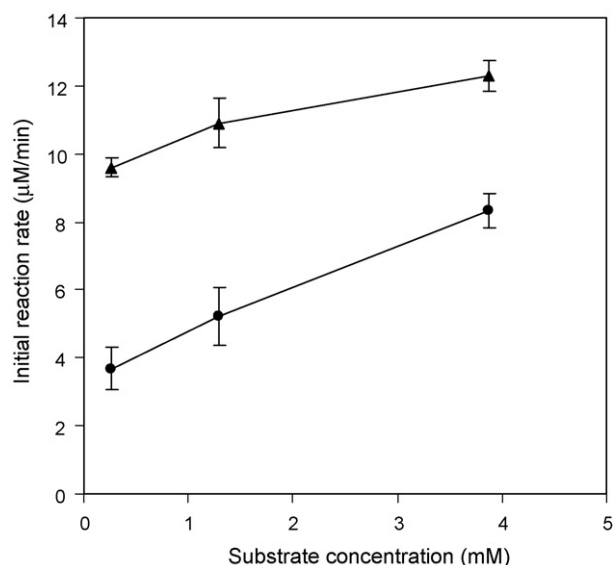


Fig. 6. Ceramide effect on the hydrolysis reaction in different sphingomyelin concentrations. (▲) Ceramide was added (0.33 mM) before the reaction. (●) Without addition of ceramide.

reaction (Fig. 6). This observation was not reported before. At lowest SM concentration (0.26 mM), the effect of ceramide concentration on the initial reaction rate was shown in Fig. 7. The effect of ceramide concentration on the hydrolysis was not linear, and certain ceramide concentrations were required to get the significant activation effect on the reaction. Since SM is an amphiphilic molecule, it is easy to congregate in the organic solvent. SM solution was cloudy with the concentration above 5 mM. In contrast, ceramide was dissolved in the organic solvent. Ceramide can interact with SM through their hydroxyl groups that are able to form hydrogen bonding between them. Therefore, the interaction between SM and ceramide result in the increase of SM solubility and diffusion rate. As a result, the addition of

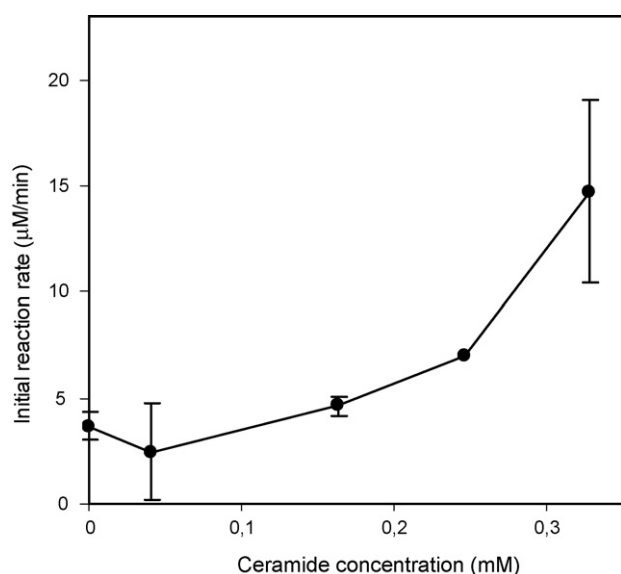


Fig. 7. Ceramide concentration effect on the hydrolysis reaction. Ceramide was added at its various concentrations before the reaction. The substrate concentration used was 0.26 mM.

Table 2

Kinetic parameters in different initial ceramide concentrations

Initial ceramide concentration (mM)	K_m (mM)	V_{max} ($\mu\text{M}/(\mu\text{g enzyme min})$)
0	1.07 ± 0.32	37.68 ± 2.91
0.16	0.21 ± 0.05	27.9 ± 1.97
0.33	0.06 ± 0.03	38.94 ± 1.38

ceramide can assist SM molecule approach to the enzyme in the interface, and the hydrolysis rate could be increased. The other possible reason is that ceramide might stabilize the enzyme in the interface, where PLC from buffer contacted SM from organic solvent to catalyze the reaction. PLC has a hydrophobic surface area near the active site [14], and ceramide is a hydrophobic compound. Therefore, through interacting with the hydrophobic surface area of PLC, ceramide might stimulate the aggregation of PLC molecule in the interface. Thus, more PLC could stay in the interface to contact SM, and the hydrolysis was consequently enhanced.

Kinetic parameters were compared in different initial ceramide concentrations (Table 2). With increasing initial ceramide concentrations, K_m decreased rapidly, and V_{max} did not change significantly. This seems to be a reversal of the effect from competitive inhibition. The big change in K_m pointed out that the addition of ceramide mainly caused the increase in the affinity of the enzyme–substrate complex. However, the specific mechanism for the product activation cannot be deduced from the result. More study is required to understand the exact principle of the activation. In large-scale productions, the product activation can be utilized to maximize the reaction rate in engineering level. In continuous stirred-tank reactor or fed-batch reactor, certain amount of the product will stay in the reactor before the new substrate joins the reaction. This will increase the reaction rate due to the product activation. Therefore, these reactors could be favoured for selection in industrial applications.

4. Conclusion

In general, the kinetic phenomenon of the selected two-phase system for the hydrolysis of SM was found quite different from previous studies. Thermal inactivation did not follow the common first order kinetics and the powder form of the enzyme showed better thermal stability than the immobilized form as commonly regarded. K_m for the two-phase system was not low comparing to the early work. However, immobilization did not change much of the K_m . Surprisingly, ceramide did not show product inhibition; instead it showed higher activation for the reaction system. The existence of ceramide reduced the K_m values of the system. This phenomenon is most likely due to the two-phase system used.

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PAPER IV

Long Zhang, Shanshan Liang, Lars I. Hellgren, Gunnar Eigil Jonsson and Xuebing Xu (2008) Phospholipase C-catalyzed sphingomyelin hydrolysis in a membrane reactor for ceramide production, *Journal of Membrane Science*, 325: 895–902



Phospholipase C-catalyzed sphingomyelin hydrolysis in a membrane reactor for ceramide production

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ABSTRACT

A membrane reactor for the production of ceramide through sphingomyelin hydrolysis with phospholipase C from *Clostridium perfringens* was studied for the first time. Ceramide has raised a large interest as an active component in both pharmaceutical and cosmetic industry. The enzymatic hydrolysis of sphingomyelin has been proven to be a feasible method to produce ceramide. In the membrane reactor constructed, the aqueous phase and the organic phase were separated by a membrane containing the immobilized enzyme, while the organic phase was continuously circulated. Among the 10 selected membranes, the enzyme immobilized in membrane RC 70PP had low immobilization efficiency, but retained the highest catalytic activity. Three immobilization methods, i.e. filtration (adsorption/entrapment), covalent binding, and cross-linking, were compared. The enzyme immobilized by filtration had the highest activity even under the low fixation level (9.4%). The optimal flow rate of the organic phase was 5 ml/min. High initial enzyme amount in the immobilization led to the decrease in the fixation level. Both the initial reaction rate and the specific activity of the enzyme increased with increasing enzyme loading, and slightly decreased after the immobilized enzyme amount over 50 µg in 9.6 cm² membrane area. The immobilized enzyme retained 16% of the original activity after five cycles. Finally, the liquid enzyme, the enzyme immobilized on particle carriers, and the enzyme immobilized in the membrane were compared. The study demonstrated the improved enzyme reusability, the fast immobilization process, the straightforward up-scaling and the combination of the hydrolysis with the product separation in the membrane reactor developed.

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1. Introduction

Ceramide (Fig. 1A) is the key intermediate in the metabolism of all complex sphingolipids. Due to its major role in maintaining the water-retaining properties of the epidermis, ceramide has great commercial potential in cosmetic and pharmaceutical industries for use in hair and skin care products. Many ceramide-containing products have already been introduced to cosmetic market, and the effect of the application of ceramide is excellent. However, chemical synthesis of ceramide is a costly and time-consuming process [1]; therefore, the development of alternative cost-efficient, high yield production methods is of great interest. Sphingomyelin (SM)

is a ubiquitous component of animal cell membranes and it is one of the major phospholipids in bovine milk. In SM, the ceramide part of the molecule is bound through a phosphodiester bridge to a choline moiety (Fig. 1B). Systematic investigation and optimization have been conducted for the enzymatic production of ceramide from SM hydrolysis [2], and phospholipase C (PLC, EC 3.1.4.3) from *Clostridium perfringens* shows high activity towards the hydrolysis reaction.

The hydrolysis is more efficient in the two-phase (water:organic solvent) system than in the one-phase (water-saturated organic solvent) system [2]. The reaction in the two-phase system is postulated to occur in the interface where the enzyme dissolved in the water phase contacts the substrate in the organic phase. An enzyme-immobilized membrane can be used to separate the two phases with their interface fixed at the membrane. Through the circulation of the organic phase, the application of membrane reactor could make it possible to produce ceramide continuously. Due to the product separation (between ceramide and phosphorylcholine) induced by the interface at the membrane (Fig. 2), enzyme

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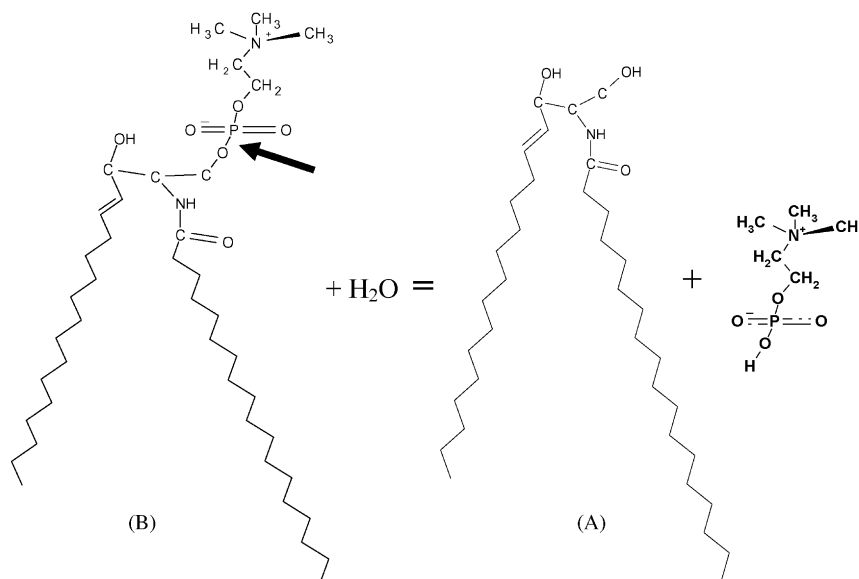


Fig. 1. The hydrolysis reaction including the structures of ceramide (A) and sphingomyelin (SM) (B). The arrow indicates the bond to be cleaved in SM for producing ceramide.

membrane reactor is able to combine bioconversion and product purification together, which reduces the number of processing steps and the operation costs. Moreover, the removal of phosphorylcholine to the aqueous phase might thermodynamically drive the reaction in the forward direction. Therefore, enzyme membrane reactor has also potential to improve the reaction rate compared with conventional methods.

Many studies have been focused on the immobilization of lipases (EC 3.1.1.3) using a variety of membrane materials and immobilization methods [3–9]. The operational stability of lipases is commonly improved by the immobilization. The lipase immobilized by covalent binding also shows higher pH and thermal stabilities than the liquid enzyme [3]. In the transphosphatidyl reaction of phosphatidylcholine, phospholipase D (EC 3.1.4.4) immobilized in a hollow fiber membrane displays high operational stability for several months [10]. However, no information from literature is available about the membrane reactor for sphingomyelin hydrolysis with PLC.

The objective of this paper was to investigate the feasibility of membrane reactor with PLC in the production of ceramide by SM hydrolysis. Phospholipase C from *C. perfringens* was immobilized in 10 membranes to test the immobilization efficiency and catalytic activity in the two-phase reaction system. Three immobilization methods, filtration (adsorption/entrapment), covalent binding and cross-linking, were compared. For characterizing SM hydrolysis in

membrane reactor, the effects from the flow rate of the organic phase and enzyme amount were evaluated, and operational stability was examined. At the end, the soluble enzyme, the enzyme immobilized on particle carriers, and the enzyme immobilized in membranes were compared to illustrate the membrane reactor's features distinct from the conventional approaches.

2. Experimental

2.1. Materials

Reference SM and ceramide from bovine brain, *C. perfringens* phospholipase C (Type I) were bought from Sigma–Aldrich Denmark A/S (Copenhagen, Denmark). Lacprodan PL-75 (phospholipids extract from milk fat globule membranes) was donated from Arla Foods Ingredients a.m.b.a (Viby J. Denmark). Bicinchoninic acid (BCA) protein assay reagent and bovine serum albumin (BSA) was obtained from Pierce (Rockford, USA). Ten membranes used in this work were described in Table 1. Standards or other chemicals were of analytic and chromatographic purity.

2.2. Sphingomyelin purification

Two gram Lacprodan PL-75 was mixed with 55 ml chloroform:methanol (90:10 by vol.). The mixture was incubated with

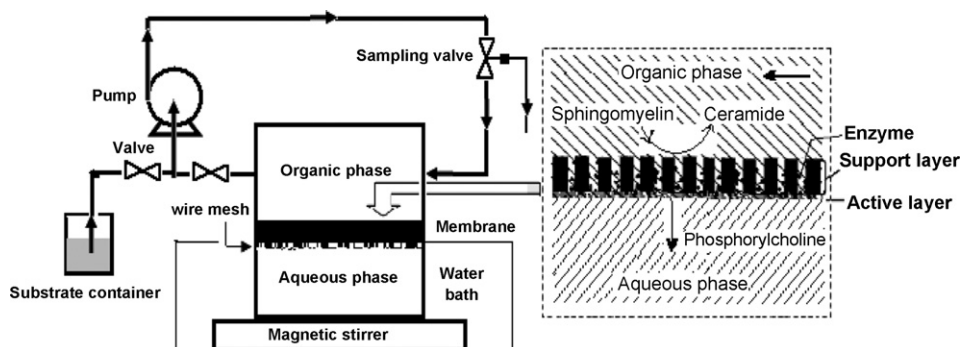


Fig. 2. Schematic drawing of the membrane reactor. The dash square showed the enzymatic hydrolysis in detail. Products, phosphorylcholine and ceramide, were separated at the interface by phase extraction during the reaction.

Table 1

Characteristics of membranes screened and the experimental results in phospholipase C immobilization.

Membrane	Manufacturer	Active layer	Support layer	MWCO ^a	Membrane state	Pre-treatment ^b	Fixation level (%)	Specific activity (μg/(mg enzyme min))	Sphingomyelin conversion (%) ^c
ETNA 01PP	Alfa Laval A/S (Nakskov, Denmark)	Hydrophilic coated PVDF	PP	1000	Dry	A	12.2	14.8	52.0
ETNA 10PP	Alfa Laval A/S (Nakskov, Denmark)	Hydrophilic coated PVDF	PP	10,000	Dry	A	62.0	6.7	49.8
ETNA 20A	Alfa Laval A/S (Nakskov, Denmark)	Hydrophilic coated PVDF	PP	20,000	Dry	A	24.6	20.0	33.4
GR 90PP	Alfa Laval A/S (Nakskov, Denmark)	PSf	PP	2000	Wet	B	20.3	25.3	80.8
GR 81PP	Alfa Laval A/S (Nakskov, Denmark)	PES	PP	10,000	Dry	A	27.1	7.9	58.2
E 1005/Job Tex	Industri-Textil Job (Kinna, Sweden)	N.A.	Woven PP	N.A.	Dry	A	61.7	6.1	65.2
E 1004/TX	Industri-Textil Job (Kinna, Sweden)	ePTFE by Pristyne®	Woven PP	N.A.	Dry	A	4.6	93.1	75.5
Hekla 10A	Alfa Laval A/S (Nakskov, Denmark)	Hydrophilic coated PES	PP	10,000	Dry	A	14.7	51.1	81.1
RC 70PP	Alfa Laval A/S (Nakskov, Denmark)	Regenerated cellulose acetate	PP	10,000	Dry	A	9.4	138.4	78.8
FS 61PP	Alfa Laval A/S (Nakskov, Denmark)	PVDF	PP	20,000	Wet	B	22.9	22.9	58.9

Abbreviations: PVDF, polyvinylidene fluoride; PP, polypropylene; PSf, polysulfone; PES, polyethersulphone; ePTFE, expanded polytetrafluoroethylene; N.A., not available.

^a MWCO: molecular weight cut-off values reported by supplier.^b Pre-treatment condition: A, soaking in water overnight; B, soaking in 96% ethanol 2 h and washing the membrane with water.^c Sphingomyelin conversion was calculated at reaction time 54 h.

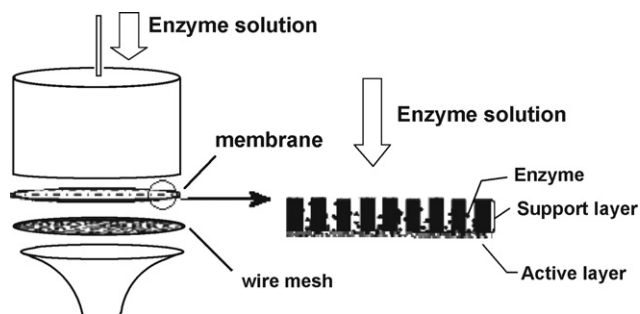


Fig. 3. Schematic view of the immobilization by dead end filtration.

7.5 ml 2 M KOH in methanol for 1.5 h at 37 °C, with shaking at 120 rpm. After adding 35 ml methanol:water (60:40), the mixture was neutralized by 1 M HCl. The organic phase was separated, washed with 15 ml methanol:water (50:50) twice and evaporated. The residual was dissolved in 8 ml chloroform, and precipitated by mixing with 20 ml acetone. After washed with 5 ml acetone three times, the precipitated SM was dissolved in 25 ml chloroform and stored at –20 °C before use. During the purification process, impurities, glycerolipids from Lacprodan PL-75, were removed by mild alkaline hydrolysis, in conditions where sphingolipids are resistant to the hydrolysis, due to the more stable amide bond between fatty acyl chain and sphingosine. The purity of SM was increased from 24.9% to 78% by the purification, and the recovery was 80%.

2.3. Enzyme immobilization

Before immobilization, dry-type membranes were soaked in MilliQ water overnight; wet-type membranes were soaked in 96% ethanol for 2 h and washed with MilliQ water. When filtration was used as immobilization method, enzyme solution (0.38 mg enzyme powder in 20 ml 0.01 M Tris(hydroxymethyl)amino-methane (Tris–HCl buffer, pH 8.6)) was filtered from the support layer to the active layer in a closed filtration cell (Fig. 3) under nitrogen (1.5–2 kg/cm²). The membrane was washed by filtering 10 ml Tris–HCl buffer twice.

With covalent binding as the immobilization method (Fig. 4), membrane RC 70PP was immersed in 10 ml 0.5 M sodium periodate solution for 90 min in dark. After washed with distilled water, the membrane was immersed in enzyme solution (0.13 mg enzyme powder in 10 ml Tris–HCl buffer) for 18 h. With cross-linking as immobilization method, membrane GR 90PP and E 1004/TX were immersed in 10 ml 2% glutaraldehyde solution for 18 h and washed with MilliQ water twice. Enzyme solution (0.13 mg enzyme powder in 10 ml Tris–HCl buffer) was filtered through the membrane twice

(the filtrate was reused in the second time). Other procedure was the same as in the filtration method.

The protein concentration of the original solution, filtrate and washing filtrate was analyzed using BCA protein assay (according to the manufactures instruction) with BSA as standard. The amount of the enzyme retained in the membrane was calculated from the protein mass balance of the solutions. Fixation level was defined as the percentage of the amount of immobilized protein.

2.4. Hydrolysis in membrane reactor

A flat sheet membrane reactor was established as the schematic diagram show in Fig. 2. The elements (made of stainless-steel) were connected by the PTFE tube (inner diameter 1.6 mm). The organic half-cell and the aqueous half-cell were cylindrical with the inner diameter 3.5 cm. They were set up vertically. The volumes of the aqueous phase and the total organic phase were 41 and 38 ml, respectively. Rubber O-rings were used in the link between components to prevent leakage. After the aqueous half-cell was filled with 0.01 M Tris–HCl buffer (pH 8.6) containing 25% ethanol and 2.5% ethyl acetate, the membrane with immobilized enzyme was fixed between the organic half-cell and the aqueous half-cell (the active layer towards the aqueous phase), with an effective area 9.6 cm². A stainless-steel wire mesh was placed below the membrane for protection. SM solution (0.318 mg/ml) in ethyl acetate:hexane (50:50) was placed in ultrasonic machine Branson 2510 (Bie & Berntsen, Rødovre, Denmark) for 5 min at 37 °C. Hydrolysis was started by pumping SM solution in the membrane elements by FMI Lab pump (Fluid Metering, Inc., Syosset, USA). Exposing of SM solution to the atmosphere was minimized to avoid evaporation. When the organic half-cell was full, the feed from the reservoir (substrate container) was stopped, and the organic phase was continuously circulated at flow rate 5 ml/min. Only the aqueous phase was kept at 37 ± 1 °C and magnetically stirred at 400 rpm. During sampling, circulation was stopped, and 100 µl reaction mixture from the organic phase was withdrawn and stored at –20 °C until analysis.

2.5. Operational stability

Enzyme solution (0.1 mg enzyme powder in 10 ml buffer) was used in the filtration. The concentration of SM in ethyl acetate:hexane (50:50) was 0.391 mg/ml. The organic and aqueous phases were changed at 24-h reaction intervals. The organic half-cell was washed with 20 ml ethyl acetate:hexane (50:50) between each runs. Other procedures were as described above.

2.6. Analysis method

Ceramide concentrations were quantified using high performance thin-layer chromatography (HPTLC) and in situ densitometry, essentially as earlier described [11]. Briefly, the HPTLC-plate (Silica gel 60, E. Merck, Darmstadt, Germany) was pre-washed through development in chloroform:methanol (2:1) in a horizontal developing chamber (Camag, Muttens, Switzerland), and the plate was activated at 120 °C for 30 min. Standards and samples were applied using DESAGA AS30 HPTLC Applicator (Sarstedt Ag & Co., Nümbrecht, Germany). Following equilibration and development with heptane:isopropanol:acetic acid (85:15:1) in the chamber, the plate was dried and sprayed with charring reagent (10% CuSO₄ in 8% H₃PO₄) until soaked. Lipids were visualized by heating the plate at 160 °C for 6 min. The intensity of the spots was determined using DESAGA CD60 HPTLC Densitometer (Sarstedt Ag & Co.) at 390 nm in the absorbance/reflection mode. The lipid mass of each component was calculated with the software DESAGA ProQuant (1.03.200, Sarstedt Ag & Co.) using a standard curve run on the

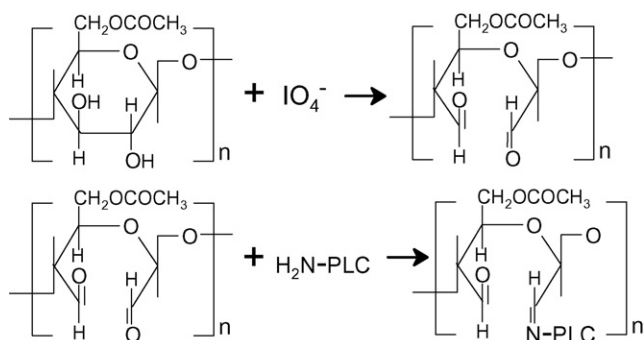


Fig. 4. The scheme of covalent binding between phospholipase C (PLC) and membrane RC 70PP.

same plate. For SM quantification of the samples from SM purification experiment, the procedure was the same except that the development solvent was chloroform:methanol:acetic acid:formic acid:water (35:15:6:2:1). Initial reaction rate was calculated from the amount of generated ceramide divided by the reaction time. Specific activity was defined as the initial reaction rate divided by the amount of immobilized protein.

3. Results and discussion

3.1. Membrane screening

The selection of a suitable membrane depends on many factors, including substrates, organic solvents, biocatalysts immobilized on the membrane surfaces, etc. Therefore, each specific reaction system requires individual optimization, in order to overcome the specific technological difficulties. Suitable membranes should be stable in used organic solvents to avoid swelling or even solubilization. In the present study, 10 different types of flat sheet membranes were tested in membrane screening experiments, with the filtration (adsorption/entrapment) as the immobilization method. All tested membranes except E 1005/Job TEX are composite membranes, which consist of the active layer with smaller pore sizes and the support layer with much larger pore sizes. The enzyme solution was filtered from the support layer to the active layer of the membrane during the immobilization. Because the pore sizes of the support layer are much larger than the active layer, the enzyme was entrapped or adsorbed on the porous structure in the membrane instead of on the membrane surface (Fig. 3). The stability should be higher than if the enzyme was loaded on the active layer, where the enzyme would be easily washed off by the water phase. These two filtration directions have been compared in literature [4], and the lipase loaded from the support layer has higher stability than loaded from the active layer. Moreover, the lipase immobilized from the support layer possibly has a better distribution profile in the membrane, which renders higher activity than the lipase immobilized on the surface of the active layer of the membrane [5].

During the filtration in the special direction, the confinement of the enzyme in the membrane depended on two factors: the size selectivity of the active layer and the adsorption of the enzyme. The fixation levels in all membranes had shown little correlation with their MWCO (Table 1). Even though MWCO of FS 61PP and ETNA 20A were 20 times as that of ETNA 01PP, there were only modest differences in their fixation levels. The molecular weight of PLC from *C. perfringens* is 43 kDa [12], while the enzyme product contained proteins at several different apparent MW, as seen in SDS-gel electrophoresis (NuPAGE, Invitrogen A/S, Taastrup, Denmark). The molecular weight of protein in the product was diverse, and small protein (around 6 kDa) had a strong signal (data not shown). Due to the diversity of the protein, the size selectivity of the membrane had less impact on the amount of total immobilized protein. In addition, MWCO of the membrane was not an absolute value but an average value, and the special filtration direction opposite to normal filtration mode probably also contributed to the little effect of membrane MWCO on the immobilization. Therefore, the size selectivity of the active layer was not a dominating factor, where the adsorption of the enzyme might be vital to the immobilization.

Adsorption of protein in membrane is affected by many parameters, such as the ionic strength and pH of the protein solution, the hydrophilic/hydrophobic nature of membrane material, and temperature, etc. [13]. Since different membranes were tested in the same conditions in the present study, the hydrophilic/hydrophobic nature of the membrane material would mainly influence the pro-

tein adsorption. The most hydrophilic membrane tested, RC 70PP (active layer was made by regenerated cellulose acetate), had lower fixation level (9.4%). This result is in agreement with the literature [5,13–14], where hydrophilic material adsorbs less protein than hydrophobic material. Membrane Hekla 10A, ETNA 01PP, ETNA 10PP and ETNA 20A had a thin hydrophilic layer (around 10 nm) coated onto the surface of the active layer. However, the coated layer was so thin, and with the special filtration direction, protein was mainly immobilized on the porous structure in the membrane instead of on the membrane surface. Hence, the hydrophilic coated layer could not have significant influence on the adsorption of protein as shown in the result.

The enzyme specific activities of the tested membranes varied from 6.1 to 138.4 $\mu\text{g}/(\text{mg enzyme min})$ (Table 1). Enzyme immobilized in the most hydrophilic membrane RC 70PP retained the highest specific activity. From the previous study [2], SM hydrolysis is more efficient in the two-phase system where the enzyme at the liquid–liquid interface has higher catalytic activity. Therefore, in the membrane reactor, the enzyme localization in relation to the interface was very important for the efficient use of the enzyme. During the reaction, the active layer was contacted with the aqueous phase, and the support layer towards the organic phase (Fig. 2). The support layers of all composite membranes were prepared from polypropylene, which is a hydrophobic material. Therefore, this layer would be filled with the organic solvent. Due to the hydrophilicity, the active layer of RC 70PP was easily immersed in water. The interface would lie deeply inside the membrane, where the enzyme mainly stayed as discussed previously. Consequently, the enzyme would be in contact with the interface, making the catalysis more efficient. In case of the hydrophobic active layer in other membranes, the organic solvent would reside in most part of the membrane, and the interface would be kept in the vicinity of (or even on) the membrane surface towards the aqueous phase. Therefore, most enzymes immobilized in the membrane would be surrounded by the organic solvent only, as the same as in the one-phase system where the activity of the enzyme is much lower in literature [2]. Lipase immobilized in hydrophilic membrane is also more active than in hydrophobic membrane in a similar two-phase membrane reactor [6]. In a series of model calculations, hydrophilic membrane, which is readily wetted both by water and polar solvents, has much thicker reaction layer (liquid–liquid interface) than hydrophobic membrane, leading to an improvement in enzyme utilization [6]. This could be the other way to explain the reason accounting for the present result.

3.2. Immobilization method

Filtration was a fast and convenient method for enzyme immobilization; however, the fixation level in most membranes was below 28%. To improve the immobilization efficiency, other immobilization methods, covalent binding (for RC 70PP) and cross-linking (for E1004/TX and GR 90PP) were investigated. The process of covalent binding for membrane RC 70PP included two steps (Fig. 4) [5]: firstly, aldehyde groups were introduced in the cellulose acetate chain by periodate oxidation; secondly, amino groups of the enzyme reacted with the aldehyde groups to form covalent bond between the enzyme and the membrane. With cross-linking as immobilization method, enzyme solution was filtered through membrane GR 90PP and E 1004/TX pre-treated by glutaraldehyde reagent, while enzyme molecules were cross-linked each other through the binding with glutaraldehyde left in the membranes [4]. This cross-linking resulted in the aggregation of enzyme molecules, and would amplify the immobilization efficiency of filtration. The active layers of GR 90PP and E 1004/TX were made from PSf and ePTFE, respectively, which are inert polymers. Therefore, covalent

Table 2

Comparison of different methods for phospholipase C immobilization in the membrane.

Membrane	Immobilization method	Fixation level (%)	Specific activity ($\mu\text{g}/(\text{mg enzyme min})$)	Sphingomyelin conversion (%) ^a
RC 70PP	Filtration	9.4	138.4	78.8
	Covalent binding	71.7	28.3	9.6
GR 90PP	Filtration	20.3	25.3	80.8
	Cross-linking	100	10.2	13.7
E1004/TX	Filtration	4.6	93.1	75.5
	Cross-linking	95.5	14.1	12.9

^a Sphingomyelin conversion was calculated at the reaction time 54 h.

binding between the membrane material and the enzyme could be excluded.

As shown in Table 2, the application of covalent binding and cross-linking increased the fixation level at least five times, indicating that they were efficient methods to improve the immobilization efficiency. However, both of the specific activity of the enzyme and the SM conversion ratio after 54 h decreased dramatically in cross-linking and covalent binding methods. The amino groups of the enzymes were covalently attached to the membrane (in covalent binding method) or to other amino groups of the enzymes with glutaraldehyde (in cross-linking process). This could alter the protein tertiary structure, causing a loss of the activity of the enzyme [5]. The other reason could be the mass-transfer limitation induced by covalent binding and cross-linking process. These two immobilization methods provided higher enzyme loading and more rigid attachment of the enzyme in the membrane than filtration. This might also increase the diffusion limitation of the substrates and products to or from the active site of the enzyme. Hence, the hydrolysis efficiency was decreased.

3.3. Filtration process

Among the three immobilization methods, filtration was proven to be the best to retain the catalytic activity of the enzyme. For the optimization of the filtration process, the effects from the number of filtration and washing were evaluated further. The above studies employed one operation of filtration and two washing steps. In the present section, enzyme solution (0.1 mg enzyme powder in

10 ml buffer) was filtered through membrane RC 70PP four times (the filtrate was reused every time), and then the membrane was washed by filtering 10 ml buffer four times (new buffer was used each time). The fixation level of the enzyme after each operation is shown in Fig. 5. The amount of the immobilized protein significantly increased in the first two batches of filtration, and reached a plateau in the following batches. Hence, two times of filtration were selected in the later study. Since the fixation level was only slightly affected by the washing times, the immobilizations were relatively stable. The purpose of washing the membranes after enzyme loading through filtration, was to remove the soluble enzyme from the membrane and make sure that the detected activity was from the immobilized enzyme only. Proteins were mainly washed out of the membrane in the first two batches of washing (Fig. 5), and two washings was thus selected for the rest of this study.

3.4. Effect of the flow rate

Through above investigations in membrane types, immobilization methods and filtration processes, suitable conditions for enzyme immobilization in the membrane were found to be: membrane RC 70PP, filtration as immobilization method, and two times of filtration and washing. These conditions were used in the following characterizations of SM hydrolysis in the membrane reactor. The effect of the flow rate of the organic phase was firstly studied in the membrane reactor. As shown in Fig. 6, the initial reaction rate increased when the flow rate increased up to 5 ml/min. This could be explained as that the increment of the flow rate enhanced the diffusive transport of SM from organic phase bulk to the reaction microenvironment and ceramide in a reverse direction.

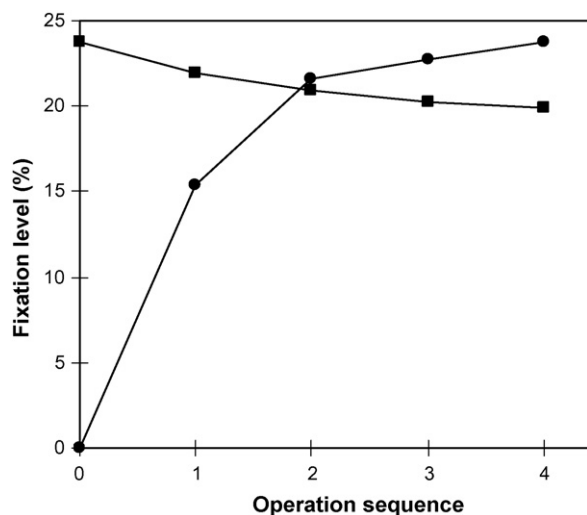


Fig. 5. The effects from the operation number of filtration (●) and washing (■) on the immobilization. Enzyme solution (0.1 mg enzyme powder in 10 ml buffer) was filtered through membrane RC 70PP four times (the filtrate was reused every time), and then the membrane was washed by filtering 10 ml buffer four times (new buffer was used each time).

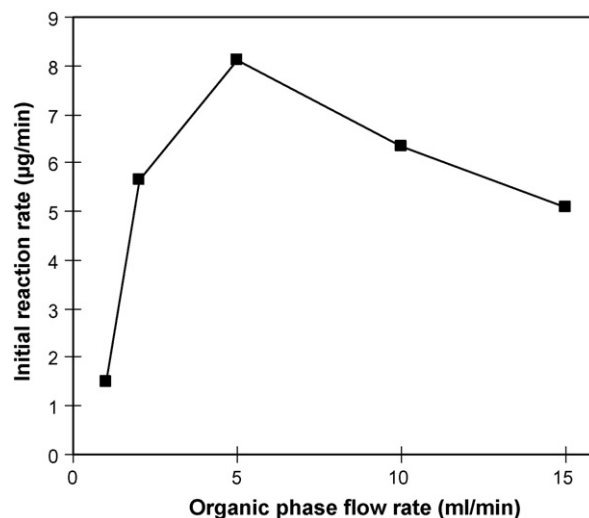


Fig. 6. The influence of the flow rate of the organic phase on the hydrolysis in the membrane reactor.

However, the initial reaction rate declined at the higher flow rate after 5 ml/min. Thus, organic phase flow rate at 5 ml/min was chosen in later study. The negative effect of the flow rate can be found from literature [7], where a polypropylene hollow fiber membrane with hydrophilic treatment is used to immobilize the lipase. High flow rate could lead the pressure rise in the organic phase. Even though the increase of the pressure would be very small in present design, it still could push the interface of the two phases in the membrane towards the water phase, hereby limiting the contact between the interface and the enzyme inside the membrane. Therefore, enzyme might be excluded from the catalytic reaction, and SM hydrolysis was inhibited. Many production plants have the facilities to control the pressures in both sides of the membrane in large reactors. At high flow rate of the organic phase, this can be balanced by increasing the pressure in the aqueous phase, thus preventing the undesirable relocation of the interface as well as the decreased reaction rate. In that case, the optimal flow rate of the organic phase would be higher than the present study.

3.5. Effect of the enzyme amount

Enzyme amount is an important factor that not only affects the reaction rate of SM hydrolysis, but also influences enzyme immobilization. Different amounts of immobilized protein were obtained by varying the initial protein amount in 10 ml buffer from 76 to 580 μg . As shown in Fig. 7A, when the initial protein amount was

increased, more protein was immobilized in the membrane by filtration. At higher initial protein amount, the increase in the amount of immobilized protein turned to be small, indicating that the membrane was saturated with loaded protein. The decrease of the fixation level with increasing the initial protein amount (Fig. 7A) also suggested that the membrane was the saturated at high initial protein amount.

Generally, when the amount of the immobilized enzyme is increased, the specific activity of the enzyme is reduced because of the decreased flexibility of enzyme conformation and the increased mass-transfer limitation of substrates and products [7]. On the contrary, both of the specific activity and the initial reaction rate increased with increasing enzyme loading, and decreased slightly only when immobilized protein was over 50 μg in the present study (Fig. 7B). When enzyme solution was filtered from the support layer to the active layer during the immobilization, some enzyme was inevitably located away from the interface and lost the activity. With low enzyme loading, the fraction of the inactive enzyme in total loaded enzyme would be higher. Therefore, the specific activity calculated from total immobilized enzyme was small with low enzyme loading. When high amount of the enzyme was immobilized in the membrane, the porous structure distant from the interface might be saturated by the enzyme, and the ratio of the inactive enzyme to total loaded enzyme possibly became small. Thus, the specific activity derived from the whole immobilized enzyme increased.

3.6. Operational stability

The improvement of the operational stability would reduce enzyme cost, and considerably encourage the industrial applications. The membrane with the immobilized enzyme was reused at 24-h intervals to examine the operational stability. The residual activity of the enzyme was significantly decreased in first two cycles, and became stable with more cycles (Fig. 8). The immobilized enzyme retained 16% of the original activity in the 6th batch. Phospholipase C immobilized on the particle carrier retains 70% of the initial activity after seven cycles [15]. However, in that study, the reaction time (20 min) for each batch and the reaction volume (2 ml) are largely different from the current study (24 h and 79 ml, respectively). Therefore, the results from two studies are not comparable. Since the enzyme immobilization in the membrane

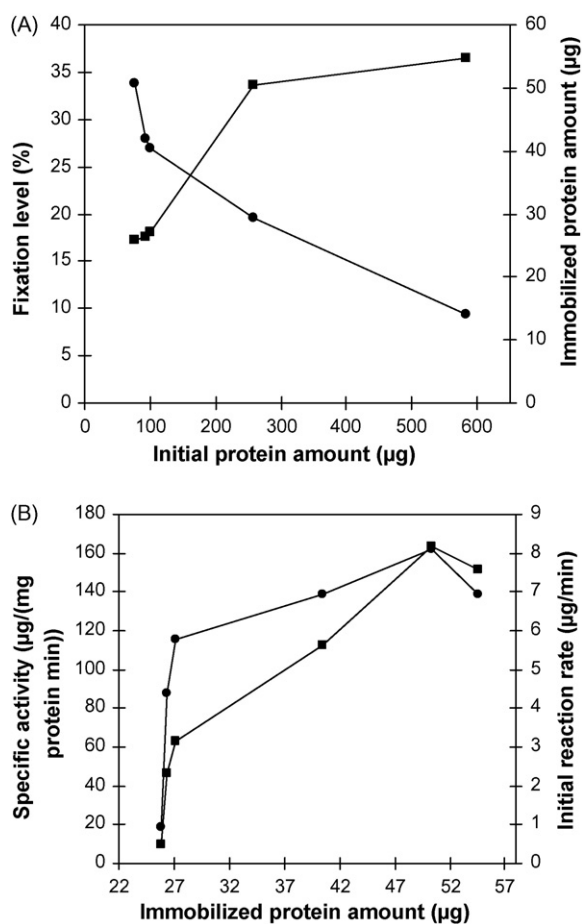


Fig. 7. The influence of the protein amount on the immobilization and sphingomyelin hydrolysis. Enzyme solution in 10 ml was used for immobilization. (A) The effect of initial protein amount on the fixation level (●) and immobilized protein amount (■); (B) initial reaction rate (■) and specific activity (●) at different amounts of immobilized protein.

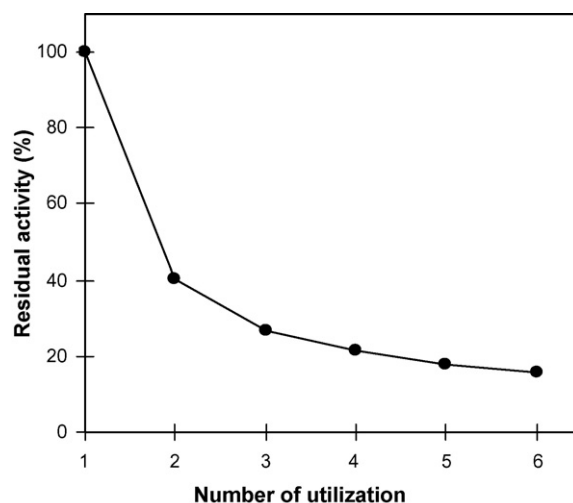


Fig. 8. Operational stability of the immobilized enzyme in membrane RC 70PP. The y-axis is the percentage of the original catalytic activity derived from the initial reaction rate.

Table 3

Comparison of different reaction systems which use the soluble enzyme, the enzyme immobilized on particle, and the enzyme immobilized in membrane.

Enzyme used for SM hydrolysis	Specific activity ($\mu\text{g}/(\text{mg enzyme min})$)	Operational stability	Time for immobilization
Soluble enzyme	3316 ^a	Difficult for reuse ^b	No immobilization
Enzyme immobilized on particle	237.7 ^a	Easy for reuse ^b	17 h ^b
Enzyme immobilized in membrane	162.3	Easy for reuse	<30 min

^a Sphingomyelin purified from Lacprodan PL-75 was hydrolyzed by methods from Ref. [17].^b Ref. [15].

was relatively stable due to its little dependence on the number of washing as shown in previous result, the enzyme loss induced by the leakage into the aqueous phase might be small, and not the main reason for the decrease of the residual activity. The loss of the catalytic activity during the repeated use might be caused by the enzyme denaturation and the twist of membrane pore structure within longer time operation.

So far, SM hydrolysis catalyzed by PLC immobilized in membrane RC 70PP was characterized through the evaluation of several important factors. In order to illustrate the membrane reactor's features distinct from the conventional reactors, some parameters were compared between different reaction systems which use the soluble enzyme, the enzyme immobilized on particle carriers, and the enzyme immobilized in membrane, individually, under the optimal conditions (Table 3). The specific activity of the enzymes immobilized both in the membrane and on the particle was much lower than the soluble enzyme. The reduction in catalytic activity of the enzyme after immobilization may be caused by different reasons such as partial denaturation, change of enzyme kinetics, partition effects and mass transport limitations [16]. In the enzyme membrane reactor, the difficulty in controlling the enzyme localization near the liquid–liquid interface, where the catalysis reaction occurred, seemed also to contribute to the loss of the hydrolysis efficiency.

The great advantage of immobilization is the reusability of the enzyme, even though the activity of the immobilized enzyme was low compared to the soluble enzyme. Enzyme immobilization in membranes through filtration was much faster than the process for the enzyme immobilization on particle carriers (Table 3), meaning that the enzyme immobilization in membrane was a convenient and easy method. Besides, up-scaling of enzyme membrane reactors are straightforward and allows continuous ceramide production. Since phosphorylcholine is soluble in the aqueous phase and ceramide soluble in the organic phase, they would be separated at the interface by phase extraction during the reaction (Fig. 2). This combination of SM hydrolysis and product separation would also be an advantage for the application of enzyme membrane reactor. In summary, based on its unique attributes shown and discussed in the present paper, the employment of enzyme membrane reactor will be attractive for the production of ceramide in large scales, and might also be feasible for a broad range of other reactions.

4. Conclusions

In the membrane reactor developed, the improved enzyme reusability, the fast immobilization process, the straightforward up-scaling and the combination of the hydrolysis with the product separation prove that SM hydrolysis in membrane reactor with PLC is a feasible approach for the production of ceramide. The enzyme immobilized in membrane RC 70PP by filtration retained the highest activity. Increasing the immobilized enzyme amount to 50 μg (in 9.6 cm^2 membrane area) resulted in high initial reaction rate and specific activity. Based on the fact that the enzyme activity is

higher in the two-phase system than in the one-phase system, the relationship between the enzyme localization and the liquid–liquid interface had significant effect on the catalytic activity, and played a crucial role during the evaluation of reaction parameters, such as membrane type, the flow rate of organic solvent and the enzyme amount, etc. Due to the intrinsic characteristics of reaction system, catalytic membrane reactor offers a good chance to improve the reaction performance, and its application in lipid area will get more and more attention in future.

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Appendix I

Effect of buffer on SM hydrolysis

Objective

In single-batch reactions, pH effect on SM hydrolysis was evaluated in a broad range from pH 3.5 to 9 (Paper I). It is difficult to find a buffer that has such broad range. We have two solutions to solve this problem: (1), using several buffers targeting to different pH ranges, respectively. Since the type of buffer often has effects on enzymatic reactions, the exact profile of pH influences can not be obtained using several buffers. (2), using a mixture buffer that has a broad pH range. In the experiments, a mixture buffer that has a broad range from pH 2 to 12 (Dawson *et al.*, 1986) was selected for pH control, and the optimal hydrolysis was at pH 8.6.

This section evaluated the effect from the type of buffer on SM hydrolysis for ceramide production, with the optimal pH at 8.6. *Clostridium perfringens* PLC was used to catalyze the reaction. Tris(hydroxymethyl)amino-methan (Tris)-HCl buffer is a commonly used buffer in enzymatic reactions. The hydrolysis performances using two buffers, Tris-HCl buffer and the mixture buffer used in Paper I, were compared, and the influence of the buffer concentration was investigated in the present study.

Materials

Standard chemicals were of the highest commercial purity available. Bovine brain SM (approx. 99%), standard ceramide ($\geq 99\%$) from bovine brain and *Clostridium perfringens* PLC (Type I, 4.6 U/mg powder, 15.8 U/mg protein from the product label) were purchased from Sigma-Aldrich Denmark A/S (Copenhagen, Denmark).

Methods

A buffer mixture with a broad range from pH 2 to 12 (Dawson *et al.*, 1986) was prepared below: citric acid, KH_2PO_4 , H_3BO_3 and diethylbarbituric acid were mixed with MilliQ water in a concentration 0.0286 M for each component. The mixture was titrated with 0.4 N NaOH to pH 8.6. To prepare Tris-HCl buffer, HCl solution was used to adjust 0.1 M Tris solution to pH 8.6.

The enzyme was dissolved in buffer containing 25 % ethanol, with a final enzyme concentration 1.9 mg protein/ml (according to the Lowry method (Lowry *et al*, 1951) using bovine serum albumin as the standard, the concentration of the protein in enzyme powder was measured to be 0.52 mg protein/mg enzyme powder). SM solution (0.1 ml of 20 mg/ml) in chloroform: methanol (2:1) was added to the reactor, and dried under nitrogen. Hereafter, 1.88 ml ethylacetate: hexane (50:50), and 0.12 ml enzyme solution were added to the reactor. The reaction was started with the addition of the enzyme solution. The reaction was performed in a sealed reactor at 37 °C using a magnetic stirrer at 450 rpm. Fifty µl aliquots of reaction mixture were withdrawn at the desired time-intervals using a syringe (Hamilton, Reno, USA). The samples were stored at -20 °C until analyzed. The samples were analyzed using the same procedure as in Paper I.

Results and discussion

The hydrolysis performances were compared between Tris-HCl buffer and the mixture buffer, and the result is shown in Fig. 1. Obviously, the hydrolysis using Tris-HCl buffer was faster than using the mixture buffer, indicating that Tris-HCl buffer was a superior buffer for SM hydrolysis. The inhibition effect of the mixture buffer was much higher than Tris-HCl buffer, even though Tris-HCl buffer has also shown the inhibition effect in the phospholipid hydrolysis catalyzed by *B. cereus* PLC (Aakre and Little, 1982). With the same mixture buffer used, SM hydrolysis in Paper I was faster than in the present study. The reason was that the enzyme amount used was less than one fifth of the amount used in Paper I.

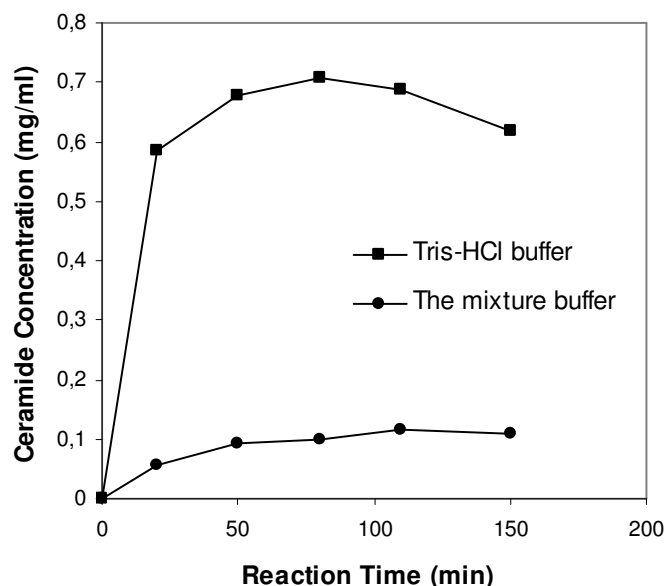


Figure 1: Comparison of the hydrolysis performances between Tris-HCl buffer and the mixture buffer.

The high hydrolysis rate using Tris-HCl buffer compared with using the mixture buffer could be explained in two aspects: Firstly, some compounds from the mixture buffer possibly had the inhibition effect on the reaction. Phosphate and citrate, which are two of the components in the mixture buffer, are able to bind conalbumin (a protein from egg white), and this binding might affect its gelation process (Oe *et al.*, 1987). Phosphate and citrate might also bind *C. perfringens* PLC, and form an ion-protein complex. Consequently, the enzyme was deactivated by this binding. Secondly, the big difference in ionic strength between these buffers could account for present phenomenon. The ionic strength of the solution is an important parameter affecting enzyme activity. The process of calculating ionic strength based on the pH and pKa is presented: The pKa values for Tris-HCl buffer, citric acid, KH_2PO_4 , H_3BO_3 and diethylbarbituric acid are 8.1, 6.4, 6.86, 9.21 and 7.98 respectively. The concentration of the ionic form of each component can be calculated using the Henderson-Hasselbalch equation

$$\text{pH} = \text{pK}_a - \log \frac{[\text{HA}]}{[\text{A}^-]} \quad (1)$$

where [HA] is the concentration of proton donor (non-ionic form), and [A⁻] is the concentration of proton acceptor (ionic form). In next step, ionic strength (I_c) for the two buffers can be calculated through below equation

$$I_c = \frac{1}{2} \sum_{B=1}^n c_B z_B^2 \quad (2)$$

where c_B is the molar concentration of ion B, z_B is the charge number of that ion, and the sum is taken over all ions in the solution. From the result, the ionic strengths for Tris-HCl buffer and the mixture buffer were 0.024 M and 0.572 M, respectively. Even though there is no clear theory about the relationship between the activity of the enzyme and ionic strength, ionic strength in the solution plays an important role to influence protein-protein and protein-substrate interactions. Change in ionic strength can alter electrostatic interactions between proteins in the solution, and strong attractive forces or repulsive forces between proteins can lead to aggregation (Vasina and Déjardin, 2004). In the present study, the ionic strength in the mixture buffer was 23 times of it in Tris-HCl buffer. The enzyme properties could be affected by the high ionic strength in the mixture buffer, which might cause the inhibition of SM hydrolysis.

In next step we evaluated the effect of the Tris-HCl buffer concentration on SM hydrolysis, using small enzyme amount (one fifth of the amount used in above study). From the result (Fig. 2), SM hydrolysis was slower when the buffer concentration was higher. The hydrolysis rate enhancement caused by a low Tris-HCl buffer concentration has been reported in literature (Aakre and Little, 1982; Little *et al.*, 1982), where the hydrolysis of phospholipids in erythrocyte ghosts and bovine brain myelin was catalyzed by *B. cereus* PLC. When the buffer concentration decreased, the ionic strength also decreased with the same proportion. As a result, the negative effect of the increase in the buffer concentration on SM hydrolysis could be caused by the change in the ionic strength.

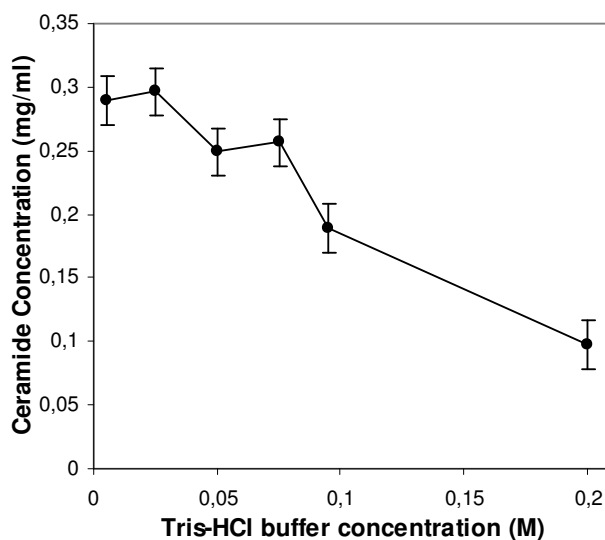


Figure 2: Effect of the Tris-HCl buffer concentration after 20 min reaction time. The enzyme amount used was one fifth of the amount used in above study. Y-error bar was calculated from the standard deviation by duplicated experiments.

Conclusion

SM hydrolysis using Tris-HCl buffer had a higher reaction rate than using the mixture buffer. Increasing the concentration of Tris-HCl buffer had a negative effect on SM hydrolysis. In later study (Paper II, III, IV), Tris-HCl buffer was used at the concentration 0.01 M.

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Appendix II

Application of magnetic particles for the immobilization of phospholipase C

Objective

As discussed in the main part of the thesis, application of enzyme immobilization has great potential due to its advantages such as the improvements of enzyme reusability and stability. Magnetic separations of immobilized enzymes have been introduced by recent studies (Guo and Sun, 2004; Schultz *et al.*, 2007). Lipase was immobilized on magnetic particles, and the immobilized enzyme was recovered by a bar magnet (Guo and Sun, 2004) or a magnetic filter cassette (Schultz *et al.*, 2007) after reaction. The immobilized lipase exhibits a good reusability in these studies. Obviously, the enzymes immobilized on magnetic particles are easy to be captured and recovered, and the process is easy to control. The aim of this study was to investigate the feasibility of applying magnetic particles on the immobilization of PLC from *Clostridium perfringens*, for the improvement of SM hydrolysis to produce ceramide.

Materials

Standard chemicals were of the highest commercial purity available. Bovine brain SM (approx. 99%), standard ceramide ($\geq 99\%$) from bovine brain and *Clostridium perfringens* PLC (Type I) were purchased from Sigma-Aldrich Denmark A/S (Copenhagen, Denmark). Magnetic particles, M-PVA N12 (Poly(vinyl alcohol) amino-activated magnetic beads) and BLM (Bacitracin-linked magnetic particles) were supplied by Chemagen Biopolymer Technologie AG (Baesweiler, Germany) and the Center for Microbial Biotechnology (in the Department of Systems Biology, Technical University of Denmark, Lyngby, Denmark), respectively.

Methods

Immobilization procedure: For magnetic particles M-PVA N12, 300 mg particles were activated by mixing with 20 ml sodium phosphate buffer (pH 7, 0.1 M) containing 12.5 % glutaraldehyde. The mixture was shaken on an over head shaker for 2 hours. The magnetic particles were separated by a magnetic bar, and washed 8-10 times with buffer. Immobilisation procedure was carried out by mixing 2 ml enzyme solution (1.14 mg protein /ml 0.1 M Tris(hydroxymethyl)amino-methan (Tris)-HCl

buffer, pH 8.6) with 30 mg magnetic particles. The mixture was shaken on an overhead shaker overnight. The solids in the mixture were separated by a magnetic bar, and 0.5 ml clear solution was taken out for analysis of the protein concentration according to the Lowry method (Lowry *et al*, 1951) using bovine serum albumin as the standard. The immobilized enzyme was washed three times with 3 ml Tris-HCl buffer (0.1 M, pH 8.6) containing 25 % ethanol and directly used in SM hydrolysis without drying.

For magnetic particles BLM, 30 mg particles were directly mixed with the enzyme solution for immobilization without any pre-activation. Other procedures were the same as described above.

Enzyme activity test: SM solution (0.1 ml of 20 mg/ml) in chloroform: methanol (2:1) was added to the reactor, and dried under nitrogen. The immobilized enzyme and 2 ml ethylacetate: hexane (50:50) was added to the reactor to start the reaction. The reactor was sealed by a cap, and kept at 37 °C with shaking at 200 rpm. Fifty µl aliquots of reaction mixture were withdrawn at the desired time-intervals using a syringe (Hamilton, Reno, USA). The samples were stored at -20 °C until analyzed. The samples were analyzed using the same procedure as in Paper I. Apparent activity was defined as initial reaction rate divided by the amount of the immobilized enzyme. Specific activity was defined as initial reaction rate divided by the amount of protein. Fixation level was the percentage of the amount of immobilized protein (estimated through subtracting the protein concentration remaining in the supernatant from the initial protein concentration).

Results and discussion

The feasibility of applying magnetic particles on the immobilization of PLC was studied in SM hydrolysis. The method of the immobilization using magnetic particles M-PVA N12 was covalent binding, with glutaraldehyde as a linker between the enzyme and the magnetic particles. Bacitracin was used as a linker in the immobilization for magnetic particles BLM. However, the exact mode of the interaction between bacitracin and the enzyme was not fully known. In the

immobilization of proteases, it is expected that bacitracin interacts with the substrate-binding sites of proteases, and both electrostatic and hydrophobic interactions are responsible for the binding between bacitracin and proteases (Petersen, 2007).

As shown in Table 1, the fixation levels of PLC immobilization using both M-PVA N12 and BLM were more than 60 %, verifying that the immobilization methods were efficient. The enzyme immobilized on BLM had a higher specific activity, 2.44 $\mu\text{g}/\text{mg}$ protein/min. Glutaraldehyde was used as a linker in the immobilization on M-PVA N12. The lower specific activity of the enzyme immobilized on M-PVA N12 could be caused by enzyme denaturation via glutaraldehyde. The specific activities in the present study were much lower than the specific activity of the soluble enzyme (64 $\mu\text{g}/\text{mg}$ protein/min, Tris-buffer 0.1 M). Although there is a great advantage for the application of magnetic particles in the immobilization, the specific activities of the enzymes immobilized on magnetic particles were too small.

Table 1: Results of PLC immobilization using magnetic particles and the activity test in SM hydrolysis.

Name of magnetic particles used	Fixation level (%)	Initial reaction rate ($\mu\text{g}/\text{min}$)	Apparent activity ($\mu\text{g}/\text{mg}$ particle min)	Specific activity ($\mu\text{g}/\text{mg}$ protein min)
M-PVA N12	64.49	0.47	0.016	0.32
BLM	60.25	3.35	0.112	2.44

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Appendix III

Production of more structurally-complicated ceramides

(The experimental results were adapted from the 10-ECT report written by students,
Kasper Skov and Kristine Weirum)

1 Introduction

Ceramides are a heterogeneous and complex group of sphingolipids containing derivatives of sphingosine bases in amide linkage with a variety of fatty acids. The main free ceramides present in the human stratum corneum matrix are classified with a number from 1-8 (Coderch *et al.*, 2003; Fig. 15 in the main part). These ceramides differ from each other by the different head groups and the fatty acid chain lengths. The fatty acids can be either α -hydroxy, ω -hydroxy or nonhydroxy fatty acids. For example, ceramide 1 and ceramide 4 contain linoleic acid, which is linked to the ω -hydroxy acids (Coderch *et al.*, 2003). Ceramide 1 is proven to be one of the most important components in skin structure, and its decrease has been found in most skin disorders (Choi and Maibach, 2005; Bouwstra and Poncet, 2006; Coderch *et al.*, 2003). Because the structure of some natural ceramides, such as ceramide 1 and ceramide 4, is too complicated, they have not been produced both chemically and enzymatically from literature. Some approaches can only synthesize simpler-structure ceramides or pseudo-ceramides (Imokawa *et al.*, 1994; Rochlin, 2002; Casey *et al.*, 1998). These more structurally-complicated ceramides have distinct fatty acid compositions from the ceramides in bovine milk SM. Therefore, they can not be produced from SM hydrolysis, which was systematically studied in other parts of the present thesis. Due to the limited resource, the potential biological functions of these compounds have not been fully unravelled in the physiological research.

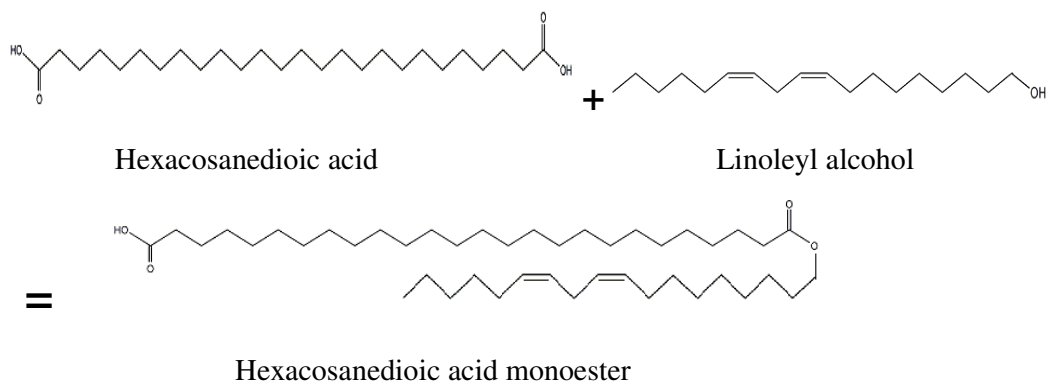
The objective of the present study was to evaluate the possibility to synthesize these more structurally-complicated ceramides, and to find a suitable method for their production. Several schemes had been proposed for the synthesis of ceramide 1 as an example. It was author's intention to produce ceramide 1 using one of proposed schemes. However, due to the requirement of much work and short time left in the Ph. D study, only a key step in the schemes, the enzymatic acylation of sphingosine, was investigated through designed experiments. Even though the product synthesized in the study was not ceramide 1, the results could give useful and essential information for further research, and the current method might be applicable for the synthesis of ceramide 1 as well.

2 Schemes for the synthesis of ceramide 1

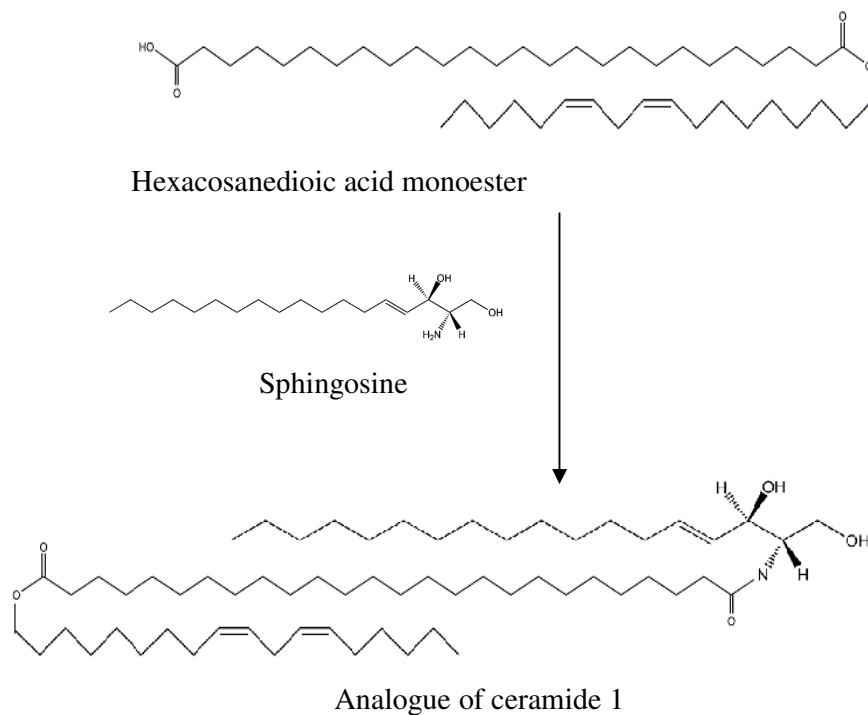
In the structure of ceramide 1, linoleic acid is linked to ω -hydroxy fatty acid with a chain length of approximately 30–32 atoms (Corderch *et al.*, 2003; Fig. 15 in the main part). In the proposed schemes of the synthesis, all the substrates were commercially available.

Scheme 1

Step 1. Hexacosanedioic acid ($C_{26}H_{50}O_4$) is partly esterified with linoleyl alcohol ($C_{18}H_{34}O$) with the catalysis by a lipase or resin. The product is hexacosanedioic acid monoester. The mono-esterification of dicarboxylic acids has been studied in literature, where a lipase (Ozaki *et al.*, 1995) or ion exchange resins (Nishiguchi *et al.*, 1999) were used as the catalyst.



Step 2. Hexacosanedioic acid monoester is applied in the acylation of sphingosine. An analogue of ceramide 1 is produced by the acylation, which is catalyzed by ceramidase. This step was investigated later through designed experiments.



There are two differences in the structure between the product in Scheme 1 and ceramide 1:

1. The fatty acyl chain length of ceramide 1 is approximately 30–32 atoms, where the chain length of the product is 26 atoms. The substrate containing the chain length of more than 26 atoms is not commercially available.
2. The direction of the ester bond is different between the product and ceramide 1. The ketone group in the ester bond of ceramide 1 belongs to linoleate chain, but the ketone group in the ester bond of the product is at the end of the fatty acyl chain.

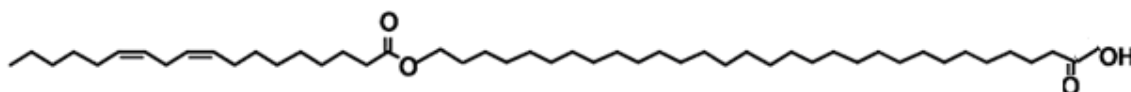
Therefore, the product in Scheme 1 is only the analogue of ceramide 1. Its biological functions are possibly similar to ceramide 1. Scheme 1 is simple compared with other schemes. However, it still requires some studies on system design, reaction optimization, analysis method, and product purification.

Scheme 2

Step 1. Triacontane-1,30-diol, $\text{HO}(\text{CH}_2)_{30}\text{OH}$, is synthesized from heptadecanedioic acid, $\text{HOOC}(\text{CH}_2)_{15}\text{COOH}$, using chemical method. The process involves at least 6 steps from literature (Drescher *et al.*, 2007).

Step 2. After the synthesis, $\text{HO}(\text{CH}_2)_{30}\text{OH}$ is partly oxidized to ω -hydroxy acid, $\text{HO}(\text{CH}_2)_{29}\text{COOH}$. The asymmetric oxidation of propane-1,3-diol to produce the corresponding ω -hydroxy acid has been achieved with a modified graphite felt electrode and sodium chlorite (Kashiwagi *et al.*, 2003).

Step 3. $\text{HO}(\text{CH}_2)_{29}\text{COOH}$ is esterified with linoleic acid. The reaction is catalyzed by a lipase. ω -hydroxy acid linoleate ester is produced in this step.



ω -hydroxy acid linoleate ester

Step 4. ω -hydroxy acid linoleate ester is used in the enzymatic acylation of sphingosine to produce ceramide 1, as in Step 2, Scheme 1.

Ceramide 1 can be synthesized from Scheme 2. However, the production of $\text{HO}(\text{CH}_2)_{30}\text{OH}$ in Scheme 2 is complicated. If we could develop a simple method for the production of $\text{HO}(\text{CH}_2)_{30}\text{OH}$, the synthesis of ceramide 1 would be easier.

Scheme 3

Step 1. Hexacosanedioic acid ($\text{C}_{26}\text{H}_{50}\text{O}_4$) is partly esterified with methanol under the catalysis by a lipase or resin (Ozaki *et al.*, 1995; Nishiguchi *et al.*, 1999). The product is hexacosanedioic acid monoester.

Step 2. The ester moiety of hexacosanedioic acid monoester is selectively reduced by lithium borohydride (LiBH_4) (Casey *et al.*, 1998). Hexacosanedioic acid monoester is converted to ω -hydroxy acid ($\text{HO}(\text{CH}_2)_{25}\text{COOH}$) in reduction reaction.

Step 3. ω -hydroxy acid ($\text{HO}(\text{CH}_2)_{25}\text{COOH}$) is esterified with linoleic acid under the catalysis by a lipase as described in Step 3, Scheme 2. ω -hydroxy acid linoleate ester is produced.

Step 4. ω -hydroxy acid linoleate ester is used in the enzymatic acylation of sphingosine to produce an analogue of ceramide 1, as in Step 2, Scheme 1.

The product of this scheme is more similar to ceramide 1 than the product of Scheme 1. The only difference from ceramide 1 is the fatty acyl chain length, which is 30–32 atoms in ceramide 1, and 26 atoms in the product of Scheme 3.

Scheme 4

Triacotanoic acid, $\text{CH}_3(\text{CH}_2)_{28}\text{COOH}$, is oxidized to ω -hydroxy acid, $\text{HO}(\text{CH}_2)_{29}\text{COOH}$. Microsomal preparations containing recombinant CYP2U1, a novel cytochrome P450 enzyme, are applied in the ω -hydroxylation of fatty acids (Chuang *et al.*, 2004). After $\text{HO}(\text{CH}_2)_{29}\text{COOH}$ is produced, the same procedures as Step 3-4, Scheme 2, are followed to produce ceramide 1. However, the preparation of microsomes containing recombinant CYP2U1 involves many steps, and is too complicated for industrial applications (Chuang *et al.*, 2004).

3 Enzymatic acylation of sphingosine to produce ceramide

3.1 Background

From above, several schemes had been proposed and evaluated for the production of ceramide 1. All schemes include an important step, the enzymatic acylation of sphingosine to produce ceramide. This step was discussed in detail, and investigated by experimental work. The selective acylation of the amino group of sphingosine has been achieved by the catalysis of crude lipase from *Pseudomonas alcaligenes* (Smeets *et al.*, 1997). However, in the investigation of several commercial lipases in the other student project in our group, no catalysis activity was detected in the acylation reaction. Therefore, we have to consider using other enzymes. Sphingolipid ceramide *N*-deacylase from *Pseudomonas sp.* TK4, alkaline ceramidase from *Pseudomonas aeruginosa*, neutral ceramidase from human intestinal and mouse liver are also capable of catalyzing the acylation reaction (Mitsutake *et al.*, 1997; Kita *et al.*, 2000; Ohlsson *et al.*, 2007; Tani *et al.*, 2000). In the present study, we used a commercial enzyme, recombinant human neutral ceramidase, to catalyze the reaction with sphingosine and radioisotope-labelled palmitic acid as substrates.

Neutral ceramidase catalyzes both the synthesis and hydrolysis of ceramides (Ohlsson *et al.*, 2007; Tani *et al.*, 2000). In ceramide synthesis (the acylation of sphingosine),

the activity of human intestinal neutral ceramidase is higher using palmitic acid as the substrate than using octanoic acid (Ohlsson *et al.*, 2007). In the study of neutral ceramidase from mouse liver, Triton X-100 is more favourable for ceramide synthesis than other detergents, i.e. sodium cholate (Tani *et al.*, 2000). Triton X-100 enhances the activity of the enzyme by about 1.5-fold at a concentration 0.1 %, but slightly decreases the activity above a concentration 0.5 % (Tani *et al.*, 2000). Through chemical modifications and mutation technique, a novel amidase sequence containing a serine residue critical for the catalytic activity has been identified in human neutral ceramidase (Galadari *et al.*, 2006), which is the same enzyme used in the present study.

3.2 Experiment

Standard chemicals were of the highest commercial purity available. Sphingosine (*trans*-D-erythro-2-Amino-4-octadecene-1,3-diol, approx. 99 %) and standard ceramide (≥ 99 %) from bovine brain were purchased from Sigma-Aldrich Denmark A/S (Copenhagen, Denmark). Recombinant human neutral ceramidase (produced by Chinese hamster ovary cells) was bought from R&D Systems Europe Ltd. (Abingdon, United Kingdom). [9, 10(n)- ^3H] palmitic acid (1.85 TBq/mmol) was purchased from GE Healthcare (Buckinghamshire, United Kingdom).

The acylation reaction conditions were mainly as described from literature (Tani *et al.*, 2000). Substrate mixture was prepared by mixing 144 nmole sphingosine and 4.8 nmole [^3H]-palmitic acid with 1.2 ml 1.0 % Triton X-100 in a 3 ml glass tube. The mixture was sonicated at ultrasonic machine Branson 2510 (Bie & Berntsen, Rødovre, Denmark) for 10 minutes at 30 °C. Fifty micro liter substrate mixture, 400 μl Tris-HCl buffer, and 50 μl enzyme solution (0.8 $\mu\text{g}/\text{ml}$, in 25 mM Tris-HCl buffer at pH 7.5) were added to the reactor to start reaction at 37 °C with shaking at 200 rpm. The initial reaction mixture included 25 mM Tris-HCl buffer, 6 nmole sphingosine, 200 pmole [^3H]-palmitic acid, 0.1 % Triton X-100 and 100 ng neutral ceramidase. After certain time, the reaction was stopped by the addition of 1.25 ml methanol, which is followed by 625 μl CHCl_3 . The mixture was shaken on a Vortex machine for 10 seconds.

For the extraction of lipids, the reaction mixture was mixed with 625 μl CHCl_3 and 625 μl 0.73 % NaCl solution, shaken on a Vortex machine, and centrifuged 3 minutes at 3000 rpm. The lower phase was transferred to a new tube, and evaporated. The sample was dissolved in 50 μl $\text{CHCl}_3\text{:CH}_3\text{OH}$ (2:1 by Vol.) and applied on a TLC plate. The TLC plate was developed by organic solvent mixture benzen: isopropanol: acetic acid (95:5:1 by Vol.) in a glass chamber for 45 min. After dried in fume hood, the plate was scanned for the radio analysis by AR-2000 radio-TLC Imaging Scanner (Bioscan Europe, Ltd., Paris, France). The standard samples were also applied and run on the same plate to help identify the compounds represented by the peak. All the experiments were triplicated, and the error bar in the figure was calculated from the standard deviation. The yield was defined in the below equation:

$$\text{Yield} = \frac{\text{peak area of ceramide}}{\text{peak area of ceramide} + \text{peak area of palmitic acid}} \times 100 \% \quad (1)$$

3.3 Results and discussion

In order to evaluate the feasibility about the production of more structurally-complicated ceramides, a key step in the proposed schemes was investigated using sphingosine and radioisotope-labelled palmitic acid as substrates. The acylation of sphingosine was catalyzed by human neutral ceramidase. It should be mentioned that the yield of ceramide synthesis was calculated based on the conversion of [^3H]-palmitic acid, which had a lower concentration than the other substrate, sphingosine. Fig. 1 shows the time course of the reaction. After 24 h reaction time, the reaction yield was around 50 %, verifying that the enzymatic acylation of sphingosine is a viable method for the production of ceramide in lab scale. The effect of the sphingosine concentration on ceramide synthesis was examined in the concentration range from 0.4 μM to 32 μM . The formation of ceramide at the reaction time 1 h increased with the rise of the sphingosine concentration (Fig. 2). Therefore, the initial reaction rate (the amount of ceramide produced / reaction time) showed a linear correlation with the sphingosine concentration. Reactions following Michaelis-Menten kinetics always show a linear relationship between the initial reaction rate and the substrate concentration at a low substrate concentration. Therefore, the present

reaction might follow Michaelis-Menten kinetics, though more experiments, especially at a high substrate concentration, are required to confirm it.

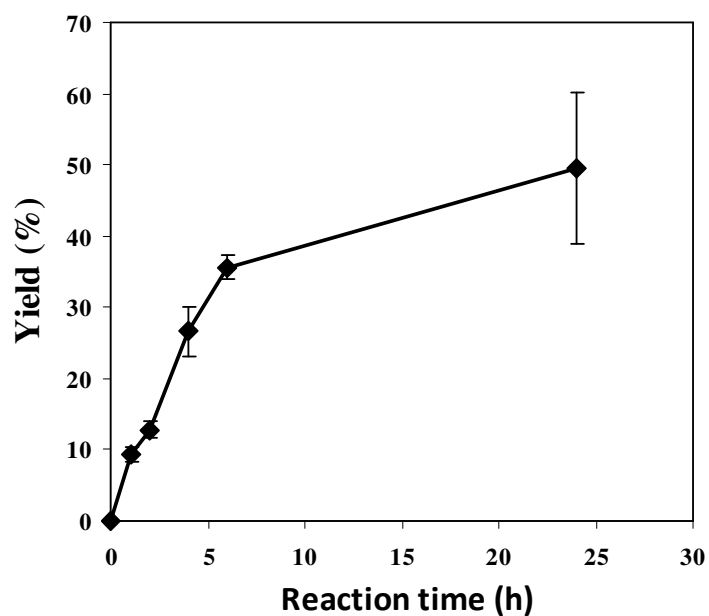


Figure 1: The time course of ceramide synthesis from sphingosine and palmitic acid.

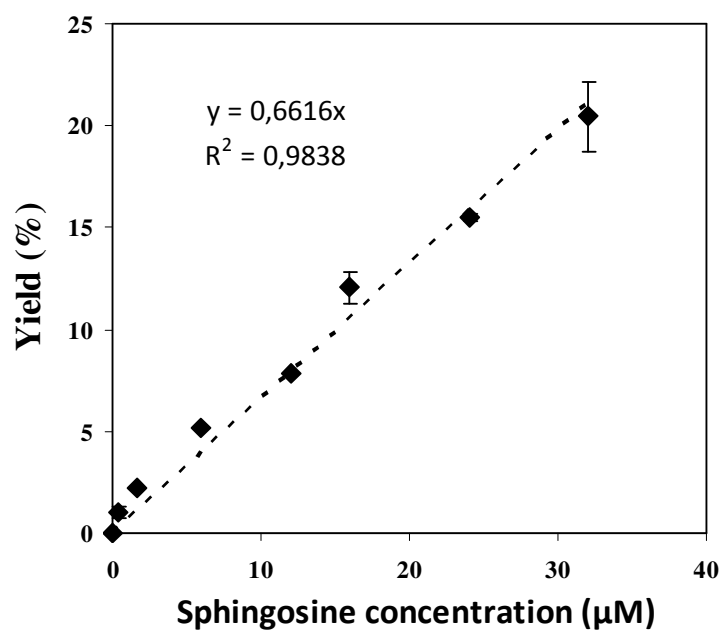


Figure 2: The effect of the sphingosine concentration on ceramide synthesis. The reaction time was 1 hour.

The present study applied a micelle-in-water system with Triton X-100 used as a detergent. Triton X-100 helped sphingosine and palmitic acid form micelles in water, making the substrate available to the water soluble enzyme. However, including the detergent in the system could also disturb product analysis and make the product separation more difficult. After the reaction, the extraction using the organic solvent was performed to separate the lipids from the reaction mixture. This increased the number of process steps. Above problems in the micelle-in-water system will become serious for the large scale production, where the substrate concentration is high. As a result, the micelle-in-water system could be replaced by an aqueous-organic biphasic system. An aqueous-organic biphasic system has been applied in sphingolipids hydrolysis catalyzed by sphingolipid ceramide N-deacylase (Kurita *et al.*, 2000). The information from this paper could be useful, although the reaction is different from the present study.

4 Summary

Due to the complicated structure, some natural ceramides, such as ceramide 1 and ceramide 4, have not been synthesized from literature. Their important functions in skin structure and in the maintenance of skin health have been recognized in many studies. With the purpose to produce these ceramides, several schemes had been proposed for the synthesis of ceramide 1. All the schemes include a key step, the enzymatic acylation of sphingosine, which was investigated further through designed experiments. Sphingosine and radioisotope-labelled palmitic acid were used in the acylation reaction catalyzed by human neutral ceramidase. A 50 % yield was achieved after 24 h reaction time. The initial reaction rate increased linearly with the rise of the sphingosine concentration. The enzymatic acylation of sphingosine was a viable method for the production of simpler-structure ceramide in lab scale. Consequently, current result would promote the later study in the synthesis of ceramide 1. However, in order to produce more structurally-complicated ceramides, proposed schemes should be investigated and compared by more experiments.

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Awards received

- 2007 Chinese Government Award for outstanding self-financed students abroad
- 2007 Lipidforum doctorate student grant for joining the 5th Euro Fed Lipid Congress and 24th Nordic Lipid Symposium, Gothenburg, Sweden, September 16-19, 2007
- 2005 The university grant for the PhD study